Neutrophils and vascular reactivity in ischaemia/reperfusion

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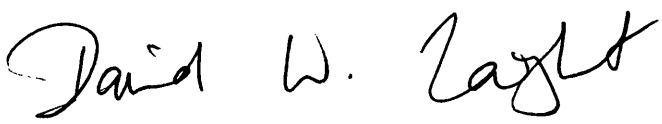

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Summary

Vasodilation to histamine in the isolated perfused rat kidney: roles of nitric oxide (NO), cyclo-oxygenase products and H_1, H_2 and H_3 receptors

The roles of nitric oxide (NO) and cyclo-oxygenase products as possible mediators of vasodilation to histamine in the preconstricted isolated perfused rat kidney have been assessed. Histamine (1-300nmoles) elicited dose-dependent reductions in renal perfusion pressure in preparations preconstricted to the same level with either potassium (30mM) or the α_1-adrenoceptor agonist, methoxamine (3μM). However, renal vasodilation to histamine (1-300nmoles) was smaller in preparations preconstricted with potassium (30mM). The selective NO synthase inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME, 0.3mM) and N^G-nitro-L-arginine (L-NOARG, 0.3mM), did not affect renal vasodilation to histamine in preparations preconstricted with either potassium (30mM) or methoxamine (3μM). Furthermore, the cyclo-oxygenase inhibitor, indomethacin (10μM), did not affect renal vasodilation to histamine in potassium(30mM)-preconstricted preparations. The H_2 receptor antagonist ranitidine (0.1-10μM) reduced and finally abolished renal vasodilation to histamine (30nmoles) in a concentration-dependent manner with a pA_2 of 6.67, estimated from a

functional inhibition curve. These results provide evidence that neither NO nor cyclo-oxygenase products play a role in a predominantly H_2 receptor-mediated vasodilation to histamine in the preconstricted isolated perfused rat kidney.

Vasoconstriction to endothelins in the isolated perfused rat kidney: effects of BQ123 and indomethacin

Endothelin-1 (ET-1), ET-2 and ET-3 (1-100pmoles) acted as equipotent vasoconstrictors in the isolated perfused rat kidney. Vasoconstriction to ETs was transient. The time to 75% recovery from the peak rise in perfusion pressure was similar for ET-1 and ET-2 (30pmoles) while that for ET-3 (30pmoles) was relatively shorter. The selective ET_A receptor antagonist BQ123 (1μM), reduced peak vasoconstrictor responses only to lower doses of ET-1 (3,10 pmoles). However, the time to 75% recovery from the peak rise in perfusion pressure to a higher dose of ET-1 (30pmoles) was reduced in the presence of BQ123 (1μM). Vasoconstriction to ET-3 (3-30pmoles) was unaffected by BQ123 (1μM). In addition, cylco-oxygenase inhibition with indomethacin (10μM) did not modulate vasoconstriction to ET-1 or ET-3 (3-30pmoles). Taken together, the evidence suggests that the ET receptors subserving vasoconstriction in the rat renal vascular bed include a population of both non-ET_A and ET_A sites. Furthermore, it is reported that cyclo-oxygenase products do not modulate rat renal vasoconstriction to ETs.

Vasoconstrictor reactivity in the isolated perfused rat kidney after renal ischaemia/reperfusion in vivo

Renal vascular resistance was unaltered in the isolated perfused rat kidney following 30min renal ischaemia with or without 15min reperfusion *in vivo*. Vasoconstrictor responses to noradrenaline (1-300nmoles) tended to be depressed during an intial dose-response series after 30min ischaemia *in vivo*. When a second dose-response series to noradrenaline (1-300nmoles) was repeated 1h later, the potency of noradrenaline was observed to have increased. No such time-dependent shift in the potency of noradrenaline occurred over a similar perfusion period in the isolated perfused rat kidney following 30min renal ischaemia and 15min reperfusion *in vivo*. In addition, renal vasoconstriction to 5-hydroxytryptamine (1-100nmoles), ET-1 (3-30pmoles), ET-3 (3-30pmoles) and KCl (110mM) was unaffected following 30min renal ischaemia *in vivo*. Taken together, the results suggest that 30min renal ischaemia *in vivo* is not associated with gross changes in vasoconstrictor reactivity or basal vascular tone *in vitro*. However, this period of occlusive renal ischaemia appears sufficient to cause a slight, reversible depression in vasoconstrictor reactivity to at least noradrenaline.

Assessment of myeloperoxidase (MPO) activity in rat renal tissue after ischaemia/reperfusion in vivo: effects of antibodies against adhesion molecules used by polymorphonucleocytes (PMNs)

It has been shown that a photometric assay of MPO derived from rat blood PMNs employing 3,3',5,5'-tetramethylbenzidine (TMB) as substrate is more sensitive than an established assay employing o-dianisidine. It was further demonstrated that rat renal tissue is capable of inhibiting peroxidase activity. This activity approached 100% when the rat renal supernate was incubated at 60°C for 2h and the assay was conducted in the presence of a 10-fold higher concentration of hydrogen peroxide (H_2O_2). Rat kidneys undergoing 45min ischaemia and 1, 3 and 6h reperfusion *in vivo*, exhibited significant increases in MPO activity, indicating PMN accumulation. Monoclonal antibodies (MoAbs) against rat intercellular adhesion molecule 1 (ICAM-1) and CD18 of $ß_2$-integrins administered both 5min before a period of 45min renal ischaemia (20µg/kg via tail vein) and at the commencement of 1h reperfusion (20µg/kg via tail vein) reduced renal PMN accumulation. However, similar treatment with the isotype-matched control murine antibody IgG_1 and an unrelated murine antibody, IgG_{2a}, also significantly reduced renal PMN accumulation. In conclusion, it can be demonstrated that the rat renal suppression of peroxidase activity may be overcome by a combination of heat

inactivation and the provision of excess assay H_2O_2. In addition, the available evidence suggests that murine MoAbs against rat adhesion molecules may exert non-specific actions in the present model of renal ischaemia/reperfusion *in vivo*.

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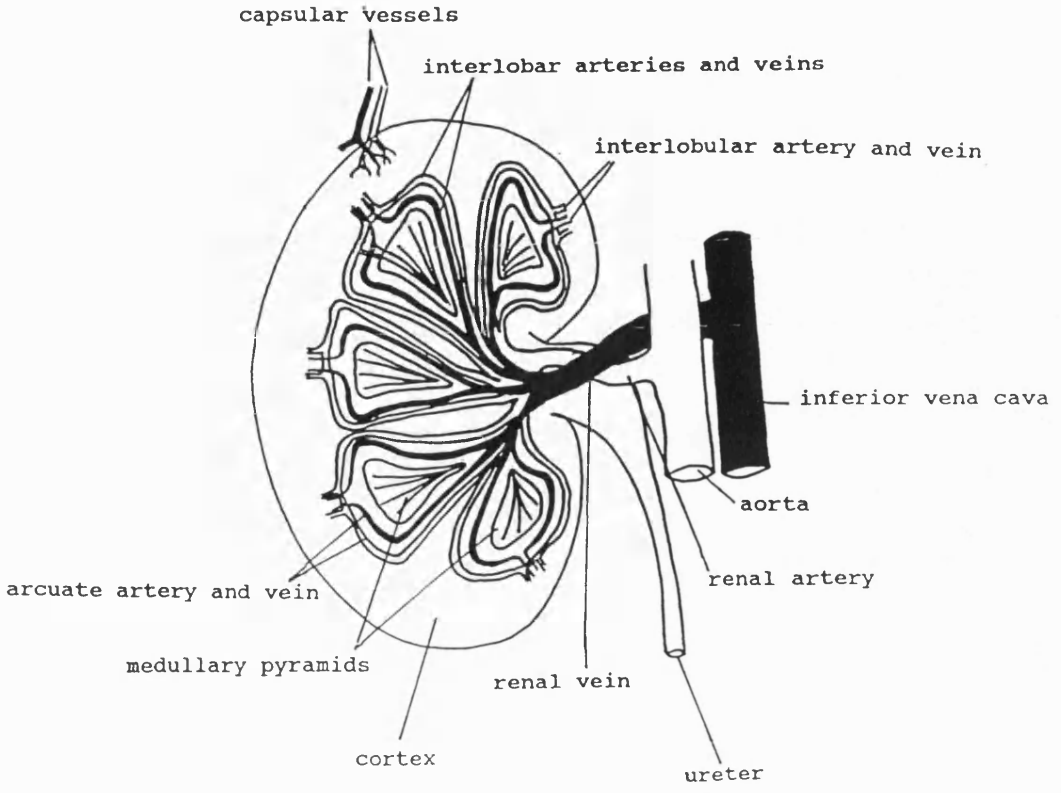
1. Introduction

1.1. The renal circulation

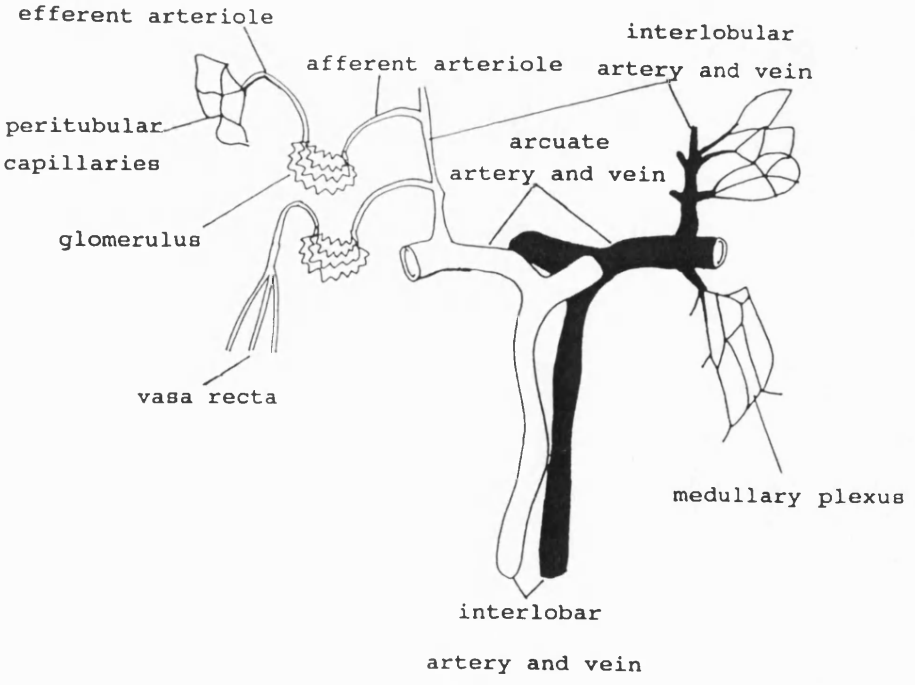
The renal artery enters the mammalian kidney at the hilum and divides into ventral and dorsal branches (Figure 1). These branches develop into interlobar arteries which ascend through the renal columns of the medulla to supply the arcuate arteries at the corticomedullary junction. Arcuate arteries then feed interlobular arteries which ascend through the cortex and engender afferent arterioles. Each afferent arteriole gives off lobules with associated capillary loops to form glomeruli. Blood drains from each glomerulus into an efferent arteriole and then into a capillary network. Superficial cortical efferent arterioles divide to produce a peritubular capillary system which supplies the capsule as well as the cortex and eventually drains into stellate and interlobular veins. Corticomedullary efferent arterioles enter the medulla and subsequently divide to provide *vasa recta*. The arterial *vasa recta* descend in bundles to terminate in capillary plexi either in the outer or inner medulla. Blood from the descending *vasa recta* drains into the ascending *vasa recta* and then into the arcuate and interlobar veins which finally converge at the hilum as the renal vein. The renal circulation of the kidney is therefore atypical in that it possesses two sets of

Figure 1. Schematic diagram showing A) gross and B) fine vasculature of the mammalian kidney

A)


capsular vessels
interlobar arteries and veins
interlobular artery and vein
inferior vena cava
aorta
renal artery
arcuate artery and vein
medullary pyramids
renal vein
cortex
ureter

B)


efferent arteriole
afferent arteriole
interlobular
artery and vein
peritubular
capillaries
arcuate
artery and vein
glomerulus
vasa recta
medullary plexus
interlobar
artery and vein

arterioles and two capillary beds. The cortex is highly vascularised and receives approximately 90-95% of total renal blood flow which itself represents approximately 10% of cardiac output (for reviews, see Vander, 1975; Brenner et al., 1976; Guyton, 1981; de Wardener, 1985; Ross et al., 1989).

A notable feature of the kidney is its autoregulatory capacity (see Vander, 1971; de Wardener, 1985). For example, renal blood flow is regulated in response to changes in arterial pressure by an intrinsic myogenic mechanism that influences the resistance of the afferent arteriole. This process in concert with another intrinsic mechanism, tubuloglomerular feedback which modulates the resistance of the efferent arteriole, regulates glomerular filtration rate. In addition, there is autoregulation of renal plasma flow in response to changes in haematocrit. The afferent and efferent arterioles in fact provide the major variable resistance vessels of the renal microvasculature in the rat (Brenner et al., 1971).

1.2. Renal ischaemia

Renal ischaemia may result from a number of diverse clinical and traumatic causes (see Schrier, 1980; Klahr, 1983; Macleod, 1984; de Wardener, 1985). For example,

cardiac failure or a sustained reduction in blood volume may lead to severe renal vasoconstriction which decreases renal blood flow. Renal vasoconstriction also occurs in response to certain poisons such as carbon tetrachloride, mercuric chloride, propylene glycol and may result from cyclosporine therapy (de Wardener, 1985). Stenotic changes in the renal microvasculature such as those arising from: arteriolar nephrosclerosis associated with malignant hypertension and systemic scleroderma; glomerular endotheliosis associated with eclampsia; the renal vascular deposition of glycoprotein in amyloidosis; and lesions accompanying renal vascular inflammation in periarteritis nodosa and glomerulonephritis, may all lead to occlusive renal ischaemia (Black, 1963; Klahr, 1983; Schrier, 1980). In renal transplantation, donor kidneys are subjected to both warm ischaemia during excision and anastomosis and cold ischaemia during storage (see Flynn, 1974). A restoration of blood flow after ischaemia may lead to clinically relevant reperfusion injury (see Hearse & Bolli, 1992; Fox, 1992). The polymorphonucleocyte (PMN) is potentially an important component of such injury, as in the microvascular and tubular dysfunction seen after renal ischaemia/reperfusion in the rat (Klausner et al., 1989; Hellberg et al., 1988).

1.3. Renal vascular reactivity after ischaemia/reperfusion

Normal or defective autoregulation (paradoxical renal vasoconstriction in response to reductions in renal perfusion pressure), depressed or enhanced vasoconstriction to renal nerve stimulation, depressed or absent vasoconstriction to noradrenaline and angiotensin II and depressed endothelium-dependent vasodilation to acetylcholine and depressed or normal vasodilation to prostacyclin have all been reported in the rat kidney following ischaemia/reperfusion *in vivo*, depending on the model and severity of ischaemia (Conger et al., 1983; 1988; 1991; Kelleher et al., 1984; Clozel et al., 1991; Matthys et al., 1983).

A raised renal vascular resistance is a common finding following occlusive ischaemia/reperfusion in the rat *in vivo* (Arendshorst et al., 1975; Paller et al., 1984; Clozel et al., 1991; Cristol et al., 1993a). Lieberthal et al. (1989) have reproduced this finding in the isolated perfused rat kidney following global non-perfusion and reperfusion with erythrocyte-rich salt solution. In their experiments, the enhanced renal vascular resistance *in vitro* was abolished by sodium nitroprusside and atrial natriuretic peptide, suggesting that vascular tone was elevated in ischaemic kidneys. The presumed inhibitors of endothelium-derived relaxing

factor (EDRF), gossypol and methylene blue (Martin et al., 1985), were found to raise renal vascular resistance to the same extent as global non-perfusion in normoperfused kidneys, suggesting the loss of a basal endothelial vasodilator role after ischaemia (Lieberthal et al., 1989). Interestingly, Cristol et al. (1993a) have suggested that vasodilator prostaglandins such as prostacyclin, help to maintain renal blood flow after ischaemia while NO release is important in the gradual recovery of renal blood flow during reperfusion *in vivo*. Spielman & Osswald (1978) have also defined a role for vasodilator prostaglandins in an attenuation of postocclusive vasoconstriction in the ischaemic/reperfused feline kidney *in vivo*. Furthermore, a role for the renal vasoconstrictors, adenosine (Sakai et al., 1980) and angiotensin II, in elevated postocclusive renal vascular resistance was proposed (Spielman & Osswald, 1978; 1979).

A similar scenario of vascular dysfunction is evident in the ischaemic myocardium. Ischaemia/reperfusion of canine coronary arteries *in vivo* results in the loss of endothelium-dependent vasodilation and enhanced contractile activity in subsequently isolated preparations *in vitro* (VanBenthuysen et al., 1987; Ku, 1982; Sobey et al., 1992); while ischaemia/reperfusion of canine myocardium leads to an increase in coronary vascular resistance and depressed vasodilator responses

to both adenosine and papaverine, indicating a deficiency in coronary vasodilator reserve (Bolli et al., 1990). In addition, reactive hyperaemia after a brief ischaemic insult is reduced (Bolli et al., 1990; Laxson et al., 1989).

1.4. Vascoconstrictor reactivity in the isolated perfused rat kidney after ischaemia/reperfusion in vivo

Attention has been focussed on the possible role of endothelins in the renal vasoconstriction accompanying ischaemic acute renal failure in the rat (Conger et al., 1983; Shibouta et al., 1990). Evidence supporting such a role is provided by the findings that the affinity of rat renal endothelin binding sites is enhanced (Nambi et al., 1992) and that the rat renal content of ET-1 is increased following ischaemia (Firth & Ratcliffe, 1992; Shibouta et al., 1990; Mino et al., 1992). An effect of ischaemia and reoxygenation to upregulate endothelin binding sites has also been reported by Liu et al. (1990) in the isolated rat heart. In addition, antibodies against ET-1 reverse postocclusive renal vasoconstriction in rats (Kon et al., 1989; Lopez-Farre et al., 1991). Endothelins are multifunctional renal peptides (see Simonson, 1993) with effects on renin release (Matsumara et al., 1988), sodium excretion (Ferrario et al., 1989; Yamada & Yoshida, 1991) and the glomerular filtration coefficient (Badr et al.,

1989), as well as glomerular filtration rate and renal vascular resistance (see Badr et al., 1989; Ferrario et al., 1989; Lopez-Farre et al., 1989; Katoh et al., 1990; Cairns et al., 1989; Takabatake et al., 1991; Gulbins et al., 1993). Accordingly, antibodies against ET-1 and ET-2 or the selective ET_A receptor antagonist BQ123, have also been shown to restore renal function and to prevent histological damage in rat models of ischaemic acute renal failure (Shibouta et al., 1990; Mino et al., 1992).

It was therefore of interest to determine how vascular reactivity to ET-1 and ET-3 together with a number of other selected vasoconstrictors in the isolated perfused rat kidney, might be affected by previous renal ischaemia *in vivo*.

1.5. Vasoconstriction to endothelins in the isolated perfused rat kidney: effects of BQ123 and indomethacin

Endothelins (ETs) and sarafotoxins (SXs) belong to a superfamily of conserved 21 amino acid isopeptides. Endothelins are found in mammalian tissues (see Simonson & Dunn, 1990) while sarafotoxins are constituents of the venom of the Israeli burrowing asp (Kloog et al., 1988). The first endothelin to be isolated, ET-1 from porcine endothelial cells, acts as a potent constrictor of smooth muscle in a variety of preparations (Yanagisawa et al.,

1988; D'Orleans-Juste et al., 1988; MacLean & McGrath, 1990). However, ET-1 may also be demonstrated to exert vasodilator effects *in vitro* in the isolated perfused rat heart (Baydoun et al., 1989) and isolated perfused rat mesentery (de Nucci et al., 1988; Warner et al., 1989) and *in vivo* in the rat hindlimb (Ohlstein et al., 1990), rat systemic vascular bed (Wright & Fozard, 1988; de Gouville et al., 1990) and feline systemic vascular bed (Lippton et al., 1988).

Endothelin isopeptides are produced by the proteolytic cleavage of 38- to 39-amino acid precursors, big endothelins, by a phosphoramidon-sensitive neutral endopeptidase known as endothelin converting enzyme (Masaki et al., 1991; Opgenorth et al., 1992; Simonson, 1993). It has been demonstrated in the anaesthetised rat that exogenous big ET-1 exerts additional renal effects to exogenous ET-1 (Pollock & Opgenorth, 1994). This finding could represent the local conversion of exogenous big ET-1 at specific sites not accessible to exogenous ET-1. The release of ET-1 from a variety of vascular and non-vascular cells is stimulated by a number of factors including shear stress, hypoxia, thrombin, thromboxane A_2, bradykinin and IL-1 (see Simonson, 1993).

Two ET/SX receptors have been characterised to date: ET_A with a rank order of binding affinities ET-1>ET-2>SX6b>ET-3 (Arai et al., 1990); and ET_B which is

isopeptide non-selective (Sakurai et al., 1990). There is some evidence for additional ET/SX receptor sites (Emori et al., 1990; Harrison et al., 1992; Martin et al., 1990; Samson et al., 1990; Watanabe et al., 1989).

Endothelins activate phospholipase C leading to the hydrolysis of phosphatidyl-4,5-bisphosphate and the formation of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Resink et al., 1988; Kasuya et al., 1989; Cioffi et al., 1991) with a concomitant activation of protein kinase C (PKC) (Lee et al., 1989). ET-1 also closes benzopyran-sensitive potassium channels (Lawson et al., 1992). Hence, smooth muscle constriction to endothelins may occur via several mechanisms, including internal calcium release, trans-sarcolemmal calcium influx through voltage-operated (Godfraind et al., 1989; Goto et al., 1989) and receptor-operated channels (Ninomiya et al., 1992; Suzuki et al., 1992), an inhibition of calcium extrusion (Wallnoeffer et al., 1989) and a sensitisation of contractile elements to calcium (for review, see Highsmith et al., 1992). An effect of ET-1 to activate voltage-operated channels (Goto et al., 1989) appears not to be due to a dihydropyridine agonist-like activity (Nakayama et al., 1991) and is conceivably mediated intracellularly by a second messenger such as PKC (see Vivaudou et al., 1988; Rasmussen et al., 1987).

In addition, endothelins activate phospholipase A_2 (Resink et al., 1989) leading to a cascade of vasoactive arachidonate metabolites in various preparations (thromboxane A_2 (de Nucci et al., 1988); prostaglandin E_2 (PGE_2) (Rae et al., 1989); prostacyclin (Lidbury et al., 1990; de Nucci et al., 1988); lipoxygenase products (Nagase et al., 1990)). Furthermore, endothelins have been shown to release platelet activating factor (PAF) (Ninomiya et al., 1992; Terashita et al., 1989), angiotensin II (Kawaguchi et al., 1990), atrial natriuretic peptide (ANP) (Hu et al., 1988), NO (de Nucci et al., 1988; Warner et al., 1989) and histamine (Ninomiya et al., 1992) in a host of preparations.

Evidence has emerged that the ET_A receptor is not an exclusive mediator of smooth muscle contraction to endothelins. Warner et al. (1992) have found *in vitro* that the selective ET_A receptor antagonist BQ123 (Ihara et al., 1992a; Nakamichi et al., 1992) fails to modulate the constrictor effect of ET-1 in the guinea-pig bronchus, rat stomach strip and rabbit pulmonary artery, but in contrast does produce blockade in the rat thoracic aorta. More recently, Cardell et al. (1993) have shown that the ET-1-induced contraction of the isolated guinea-pig trachea is insensitive to the novel selective ET_A receptor antagonist, FR13917. *In vivo*, Bigaud & Pelton (1992), Cristol et al. (1993b) and McMurdo et al. (1993) have shown that BQ123 largely depresses but does not

abolish the pressor effects of endothelins in the anaesthetised rat. In addition, Douglas et al. (1992) have not been able to abolish the ET_A-mediated secondary pressor effect of ET-1 (Bigaud & Pelton, 1992) using BQ123, without compromising the ET_B-mediated initial depressor effect (Bigaud & Pelton, 1992) in the anaesthetised rat.

Direct evidence that the ET_B receptor can subserve vasconstriction to endothelins/sarafotoxins was provided *in vivo* by the pressor effects of the reputed ET_B-selective agonists SX6c (Williams et al., 1991) and [$Ala^{1,3,11,15}$]ET-1 (BQ3020) (Ihara et al., 1992b; Saeki et al., 1991) in the anaesthetised (Bigaud & Pelton, 1992; Cristol et al., 1993b; Clozel et al., 1990) and pithed rat (Williams et al., 1991). Moreover, *in vitro*, Ihara et al. (1992b) have demonstrated constriction of the isolated, endothelium-denuded rabbit pulmonary artery to the ET_B selective agonist BQ3020. BQ3020 displayed a similar potency to both ET-1 and ET-3 as a constrictor and responses were unaffected by BQ123. There is also evidence linking the ET_B receptor to constriction in the porcine coronary artery (Ihara et al., 1992a), where ET_A receptors are also present. These findings clearly refute the notion that the ET_B receptor is dedicated to dilation in the vasculature (Takayanagi et al., 1991). In contrast, ET-1-elicited contractions of the isolated dog (Douglas et al., 1993) and guinea-pig (Cardell et al.,

1993) pulmonary artery have indeed been shown to be mediated predominantly by ET_A receptors.

Very recent evidence from several *in vivo* studies in the rat indicates that renal vasoconstriction to ETs and SXs is mediated by ET_A and non-ET_A, ET_B-like ET/SX receptors. Both Cristol et al. (1993b) and Bigaud and Pelton (1992) have shown only partial inhibition of renal vasoconstriction to endothelins in the anaesthetised rat with the selective ET_A receptor antagonist BQ123. Indeed, Pollock & Opgennorth (1993) have reported that ET-1 elicited renal vasconstriction is unaffected by BQ123 in the anaesthetised rat. In direct support of a role for ET_B receptors in the renal vasoconstriction to ETs/SXs in the anaesthetised rat, Bigaud & Pelton (1992), Clozel et al. (1993) and Cristol et al. (1993b) have demonstrated increases in renal vascular resistance to the reputed ET_B-selective agonists, SX6c and BQ3020. Williams et al. (1991) have also shown renal vasoconstriction to SX6c in the pithed rat. Interestingly, the blood pressure-elevating effect, but not the renal vasoconstrictor effect, of SX6c was found to be partially diminished by BQ123 in the anaesthetised rat by Cristol et al. (1993b). This raises the possibility that reputed ET_B-selective agonists may exert additional effects. Furthermore, a non-ET_A, non-ET_B, SX6c/ET-3-selective receptor, originally described in the porcine coronary artery and coupled to constriction (Harrison et al., 1992), may

conceivably mediate some of the direct effects of SX6c. Consistent with an ET_B profile, Cristol et al. (1993b) determined the rank order of potency of endothelins and sarafotoxins as renal vasoconstrictors to be ET-1=ET-3=Sx6b=SX6c in the anaesthetised rat. Inconsistent with this ET_B profile, however, is the failure of the nonselective ET_B receptor antagonist PD142893 (Hingorani et al., 1992) to inhibit renal vosoconstriction to ET-1 in the anaesthetised rat (La Douceur et al., 1992). In the same preparation, PD142893 was reported to selectively inhibit the initial depressor response to ET-1 and to inhibit regional vasodilation in the hindlimb.

Such findings in the rat *in vivo* arguing for a mixed population of ET_A and possibly ET_B receptors in renal vasoconstriction to endothelins are in contrast to studies in other species. For example, the selective ET_A receptor antagonists BQ153 (Fukuroda et al., 1992) and BQ123 (Ihara et al., 1992a; Nakamichi et al., 1992) have been found to substantially reduce vasoconstriction to ET-1 in the porcine kidney *in situ* (Cirino et al., 1992) and the isolated perfused rabbit kidney (Télémaque et al., 1993), respectively. In addition, the relative potency of endothelin isopeptides as vasoconstrictors in the isolated perfused rabbit kidney was ET-1>ET-2>ET-3 while the ET_B agonists BQ3020 and IRL1620 were without effect (Télémaque et al., 1992; 1993).

In the present study, the receptors subserving vasoconstriction to endothelins in rat isolated perfused rat kidney were investigated by establishing a rank order of potency for endothelin isopeptides and by examining the sensitivity of vasoconstriction induced by ET-1 and ET-3 to BQ123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]), a potent and selective cyclic pentapeptide ET_A receptor antagonist (Ihara et al., 1992a; Nakamichi et al., 1992). In view of the fact that endothelins could conceivably modulate their direct vasoconstrictor effects indirectly via the release of vasoactive eicosanoids, a role for cyclo-oxygenase in the renal vascular reactivity to ET-1 and ET-3 was additionally examined using indomethacin.

1.6. Vasodilation to histamine in the isolated perfused rat kidney as an assessment of EDRF(NO) release?

Histamine acts as a renal vasodilator in the preconstricted rat isolated perfused kidney (Bhardwaj & Moore, 1988). On the basis of experiments with metyrapone, nordihydroguaiaretic acid and gossypol, postulated inhibitors of EDRF synthesis, methylene blue, a presumed antagonist of the effect of EDRF, and CHAPS, a detergent that removes endothelial cells lining blood vessels, Bhardwaj & Moore (1988) proposed that renal vasodilation to histamine was dependent on an intact endothelium and the biosynthesis of EDRF, first described

by Furchgott & Zawadzki in 1980. EDRF has since been identified as NO by Palmer et al. (1987) which is derived from L-arginine by NO synthase (Palmer et al., 1988).

There is plentiful evidence for the role of a relaxing factor(s) derived from the endothelium in the relaxation of vascular smooth muscle to histamine. In the isolated rat thoracic aorta (Van de Voorde & Leusen, 1983a), guinea-pig pulmonary artery (Satoh et al., 1984) and human pulmonary artery (Ortiz et al., 1992), H_1 receptor dependent relaxation was abrogated by mechanical removal of the endothelium. Similarly, H_2 receptor dependent vasodilation of the rabbit cerebral artery was circumvented by endothelial denudation (Sercombe et al., 1986). In addition, mechanical removal of the endothelium in the dog mesenteric artery reduced the vasodilator response to histamine (Toda, 1984). Indeed, Hide et al. (1988) have demonstrated the presence of H_1 receptors on both the endothelium and vascular smooth muscle of the guinea-pig aorta and there is evidence of site dependent relaxant and constictor effects of H_1 receptor stimulation in the rabbit cerebral artery (Kim et al., 1986) and guinea-pig pulmonary artery (Satoh et al., 1984). Toda (1984) was able to block vasodilation of the dog mesenteric artery to histamine with indomethacin and suggested that prostacyclin release from both endothelial and vascular smooth muscle sources was involved. In the human pulmonary artery (Ortiz et al., 1992), endothelium-

dependent vasodilation to H_1 receptor stimulation was abolished by a combination of indomethacin and L-NAME or L-NOARG. The endothelium-dependent vasodilation of the rabbit cerebral artery to H_3 receptor stimulation was similarly attenuated by indomethacin and dexamethasone, suggesting prostacyclin release, and L-NAME and N^G-monomethyl-L-arginine (L-NMMA), indicating NO release (Kim et al., 1992).

Given the availability of agents which selectively inhibit the biosynthesis of NO such as L-NAME and L-NOARG (Rees, 1990; Moore et al., 1990), it was important to investigate whether EDRF(NO)-dependence for renal vasodilation to histamine in the preconstricted rat isolated perfused kidney could be demonstrated with these more selective tools. In addition, given the ability of histamine to stimulate the release of prostacyclin from cultured human umbilical endothelial cells (Baenziger et al., 1981; McIntyre et al., 1985), a role for vasodilator cyclo-oxygenase products in the mediation of renal vasodilation to histamine was investigated using indomethacin.

1.7. Further aspects of histamine pharmacology

Histamine is a biogenic amine with diverse pharmacological actions which are mediated by three known

classess of receptors: H_1-H_3 (for review, see Hill 1990). A typical response allied to H_1 receptor stimulation concerns the contraction of smooth muscle (pig trachea (Driver et al., 1987);, guinea-pig ileum and trachea (Arunlakshana and Schild, 1959; Dews and Graham, 1946); guinea-pig pulmonary artery (Satoh et al., 1984); rabbit cerebral artery (Kim et al., 1986)). Postcapillary venular endothelial cells may also be contracted in response to H_1 receptor stimulation (Majno et al., 1969). However, H_1 receptor stimulation may also lead to the relaxation of smooth muscle in a host of isolated tissues (see above), including the methacholine-preconstricted guinea-pig trachea (Undem et al., 1987). In this study, histamine-mediated tracheal relaxation was independent of catecholamine release and the epithelium and sensitive to inhibition by indomethacin (Undem et al., 1987).

The stimulation of H_1 receptors is associated with the activation of phospholipase C and phosphoinositide hydrolysis, leading to the formation of IP_3 and DAG in a variety of tissues including endothelial cells (Lo & Fan, 1987), vascular (Lonchampt et al., 1988) and visceral smooth muscle (Jafferji & Michell, 1976). For reviews of IP_3 and DAG as second messengers, see Berridge et al. (1984; 1993).

A typical response allied to H_2 stimulation concerns the relaxation of smooth muscle (guinea-pig lung parenchyma (Foreman et al., 1985); rabbit mesenteric artery (Reinhardt & Ritter, 1979); rabbit cerebral artery (Sercombe et al., 1986); feline cerebral artery (Edvinson et al., 1983); human temporal artery (Ottosson et al., 1989)). The stimulation of H_2 receptors is associated with the activation of adenyl cyclase in a variety of tissues including vascular smooth muscle (Reinhardt & Ritter, 1979), airway smooth muscle (Eyre & Chand, 1982) and cardiac muscle (Hattori et al., 1991). This leads to the intracellular accumulation of cyclic adenosine monophosphate (cAMP). Within smooth muscle, cAMP activates protein kinase-dependent phosphorylation, which leads to relaxation through the inhibition of myosin light chain kinase activity (Silver & Still, 1982), membrane hyperpolarisation (Fujiwara et al., 1988; Kume et al., 1989) and internal calcium sequestration (Mueller & Van Breeman, 1979).

The H_3 receptor was first described as a presynaptic autoreceptor in the rat cerebral cortex (Arrang et al., 1983) and has since been described in the human cerebral cortex (Arrang et al., 1988), the guinea-pig iluem (Trzeciakowski, 1987), guinea-pig airways (Ichinose et al., 1989) and the guinea-pig mesenteric artery (Ishikawa & Sperelakis, 1987). The first case of a postjuntional H_3 receptor was reported in the rabbit cerebral artery (Kim

& Oudart, 1988). Documented responses to H_3 receptor stimulation include the inhibition of neurotransmitter histamine synthesis and release (Arrang et al., 1987), the inhibition of sympathetic (Ishikawa & Sperelakis, 1987), cholinergic (Ichinose & Barnes, 1989) and non-adrenergic non-cholinergic transmission (Taylor & Kilpatrick, 1992) and arterial vasodilation (Kim et al., 1988; 1992). The second messenger system(s) associated with H_3 receptors remains to be elucidated.

It was therefore of additional interest to explore the relative contributions made by the three documented classes of histamine receptor to renal vasodilation to histamine in the isolated perfused rat kidney.

1.8. Polymorphonucleocytes (PMNs) and vascular dysfunction after ischaemia/reperfusion

A feature commonly associated with the reperfusion of ischaemic tissue is a histologically demonstrable accumulation of PMNs, as in the ischaemic/reperfused dog and rat myocardium (Romson et al., 1983; Engler et al., 1986; Hale & Kloner, 1991; Smith III et al., 1988) and canine gracilis muscle (Walden et al., 1990). The ischaemic/reperfused rat kidney *in vivo* is no exception with a histologically verifiable PMN accumulation reported to occur in the inner stripe of the outer

medulla within 5 min of reperfusion after 45 min renal artery occlusion (Willinger et al., 1992). Within 2h reperfusion, PMN accumulation was predominant in the cortex and outer stripe of the outer medulla (Willinger et al., 1992). Reperfusing PMNs have been implicated in both myocardial infarction (Romson et al., 1983; Engler et al., 1986; Bednar et al., 1985; Smith III et al., 1988), coronary vascular endothelial injury (Sheridan et al., 1991; Kloner et al., 1991), ischaemic bowel injury (Hernandez et al., 1987) and skeletal muscle contractile dysfunction following ischaemia/reperfusion (Carden et al., 1990; Walden et al., 1990). While the benefits of PMN depletion to postocclusive renal function in the rat are equivocal (Thornton et al., 1989; Paller et al., 1986; Klausner et al., 1989), this intervention has been reported to improve renal blood flow after 45 min occlusive renal ischaemia/reperfusion in the rat *in vivo* (Klausner et al., 1989).

A postocclusive perfusion defect located in both the outer and inner medulla has been well documented in the rat kidney *in vivo* and leads to the phenomenon of "no reflow" after ischaemia. The perfusion defect involves intravascular congestion with erythrocytes (Vetterlein et al., 1986; Mason et al., 1987) and probably also PMNs (Hellberg et al., 1990) and possibly in addition, tubular epithelial swelling with subsequent encroachment on the lumens of peritubular capillaries and the venous *vasa*

recta (Yamamoto et al., 1984). An increase in microvascular permeability and a loss of cell volume regulation during ischaemia leads to renal oedema and heamoconcentration (Flores et al., 1972; Vetterlein et al., 1986). Interestingly, fibrin deposition has been reported to contribute to ischaemic renal oedema in the rat *in vivo* (Druid & Rammer, 1992) while thrombin is not thought to play a role in medullary erythrocyte congestion (Mason et al., 1987). The "no reflow" phenomenon has also been described in the ischaemic canine myocardium *in vivo* where it has been variously attributed to straightforward tissue necrosis (Darsee & Kloner, 1980), capillary plugging with PMNs (Engler et al., 1983) and myocardial oedema (Kloner et al., 1974). A prime role for PMN vascular plugging was proposed in the globally ischaemic isolated perfused rat heart where exogenous, reperfusing PMNs enhanced coronary vascular resistance by reducing capillarity (Reynolds & McDonagh, 1989). There is evidence from the ischaemic canine myocardium that the expression of the "no reflow" phenomenon is related to the period of ischaemia (Kloner et al., 1974).

The role of PMNs in renal vascular dysfunction following ischaemia/reperfusion may not be restricted to rheological capillary plugging and the "no reflow" phenomenon. PMNs are capable of producing tissue damage by the release of cytotoxic species such as oxygen free

radicals and degradative enzymes and furthermore release vasoactive arachidonate metabolites (for reviews, see Mehta et al., 1988; Harlan, 1987; Korthuis & Granger, 1993; Downey, 1990; Rowe et al., 1984; Werns & Lucchesi, 1988; Kukreja & Hess, 1992). PMNs may therefore modulate vascular reactivity via effects on vascular smooth muscle integrity, microvascular permeability and the metabolism of endothelial vasoactive autacoids in addition to direct effects through the production of vasoactive mediators.

The salutary effect of PMN depletion in the rat on postocclusive renal blood flow reported by Klausner et al. (1989), was associated with reduced renal venous levels of thromboxane B_2. Complimentary to this finding, the thromboxane A_2 synthase inhibitor, DP-1904, has been demonstrated to attenuate the postocclusive rise in renal vascular resistance in the anaesthetised rat (Masumara et al., 1991). Other direct vasoconstrictors elaborated by PMNs include platelet activating factor (Tippins et al., 1992) and peptido-leukotrienes. Angiotensin II and endothelin-1 may also be formed by PMN-mediated, enzymatic cleavage of angiotensinogen (Wintroub et al., 1984) and big-endothelin-1 (Sessa et al., 1991), respectively. In addition, canine PMNs may elicit endothelium-dependent constriction of the isolated canine femoral artery via the endothelial uptake and metabolism of PMN-derived leukotriene A_4 to peptido-leukotrienes (Gonzales et al., 1992). Furthermore, PMNs may interact

with platelets to promote vasoconstriction (Sessa & Mullane, 1990).

Accounting for an indirect vasoconstrictor effect of PMNs is their potential to inactivate NO via the production of the superoxide anion (Gryglewski, 1987; Tsao & Lefer, 1990). This is thought to account for the endothelium-dependent and superoxide dismutase-sensitive constriction of the isolated rabbit aorta to PMNs (Ohlstein et al., 1989). Furthermore, the presence of superoxide anion is responsible for the PMN-mediated loss of endothelium-dependent vasodilation in the isolated bovine and rat mesenteric artery (De Kimpe et al., 1993; Karasawa et al., 1991). Another aspect of PMN-impaired endothelial vasodilator function is an enhanced reactivity to vasoconstrictors (De Kimpe et al., 1992). Despite these pro-vasoconstrictor effects, there is some evidence that at least human and rat PMNs may also elaborate a modulatory vasorelaxing factor similar to NO (Mehta et al., 1989; Rimele et al., 1987) while rat PMNs in addition may produce endothelium-dependent vasodilation (Grossman & Zambetis, 1989).

The finding by Augustin & Lutz (1991) that lipid peroxidation accompanies rat renal occlusive ischaemia/reperfusion *in vivo* coupled with the demonstration by Paller et al. (1984) that oxygen free radicals contribute to postocclusive renal dysfunction

and a raised renal vascular resistance in the rat *in vivo*, may admit a role for PMNs as a potential source of such radicals. In support of this, Linas et al. (1988) have shown that exogenous, reperfusing PMNs enhanced renal dysfunction in the isolated perfused rat kidney previously made ischaemic by renal artery occlusion *in vivo* and that the oxygen free radical scavengers dimethylthiourea and catalase were protective. Extensive endothelial damage was recorded after renal ischaemia and reperfusion both *in vivo* (Paller et al., 1984) and *in vitro* (Linas et al., 1988), while the latter group reported damage to vascular smooth muscle specific to reperfusion with exogenous PMNs *in vitro*. It was noted that despite these findings, basal renal vascular resistance was unaltered by reperfusing PMNs (Linas et al., 1988). However, it has been shown in both the isolated perfused rabbit and rat heart that activated PMNs may indeed raise coronary vascular tone via the production of oxygen free radicals (Gillespie et al., 1986; Semb et al., 1989). One possibility is that oxygen free radicals stimulate the production of thromboxane A_2 (Tate et al., 1984). Linas et al. (1992) have subsequently found that rat kidneys made mildly ischaemic *in vivo* can activate reperfusing primed PMNs *in vitro* to release oxygen free radicals and elastase, leading to impaired renal function. It was previously recorded by Welbourn et al. (1991) that PMN-derived oxygen free radicals and elastase may synergise to produce

permeability oedema in the lung after hindlimb ischaemia in the rat.

1.9. PMN infiltration

The infiltration of tissue by PMNs proceeds by three major contiguous events: rolling adhesion of PMNs to the vascular endothelium or margination; shear-stress resistant or firm adhesion of PMNs; and PMN migration across the endothelial monolayer. The endothelium plays an active role in PMN infiltration from the recruitment of PMNs via the production of chemotactic cytokines such as interleukin-8 (IL-8) and melanoma growth-stimulating activity (MGSA) to the development of a proadhesive state (for reviews, see Lefer & Lefer, 1993; Mantovani et al., 1992; Pober & Cotran, 1990). Adhesion molecules of the selectin family, the β_2-integrin subfamily and the immunoglobulin family orchestrate these events (for reviews, see Springer (1990), Carlos & Harlan (1990), Harlan & Liu (1992), Mackay & Imhof (1993), Zimmerman (1992), Kayestha (1992) and Shimizu (1992)).

The β_2-integrins are leukocyte surface glycoprotein heterodimers consisting of a common 95KDa ß-subunit (CD18) and one of three variable α-subunits, designated CD11a (177KDa), CD11b (165KDa) and CD11c (150KDa) (Albelda & Buck, 1990). The combinations engender LFA-1

(CD11a/CD18), Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18). The immunoglobulin, intercellular adhesion molecule 1 (ICAM-1) acts as an endothelial ligand for leukocyte LFA-1 and Mac-1 (Diamond, 1990; Patarroyo & Makgoba, 1989; Smith et al., 1988). The importance of CD18 and ICAM-1 *in vitro* to the firm adherence of both stimulated PMN to quiescent endothelial cells (Lo et al., 1989; Mulligan et al., 1992; Smith et al., 1989; Zimmerman & McIntyre, 1988) and unstimulated PMN to activated endothelial cells (Furie et al., 1991; Luscinkas et al., 1989; Pohlman et al, 1986) has been amply demonstrated. Moreover, Argenbright et al. (1991) have shown that the adherence of stimulated PMN to the vascular endothelium of the rabbit mesenteric vasculature *in situ* is CD18- and ICAM-1-dependent. Furthermore, monoclonal antibodies (MoAbs) against CD18 have been reported to reduce tissue PMN infiltration both in response to the intradermal injection of a range of inflammatory mediators (Arfors et al., 1987; Rampart & Williams, 1988) and following ischaemia and reperfusion, as in the dog myocardium (Tanaka et al., 1993), rabbit lung (Horgan et al., 1990), cat myocardium (Ma et al., 1991) and cat mesentery (Oliver et al., 1991). *In vitro*, Barton et al. (1989) have demonstrated that the infiltration by PMNs in the isolated inflamed rabbit lung is attenuated by MoAbs against CD18 and ICAM-1 adhesion molecules. In addition to the role of β_2-integrins and ICAM-1 in firm adhesion, there is also evidence that

these adhesion molecules facilitate transendothelial migration (Kavanaugh et al., 1991; Parkos et al., 1991; Smith et al., 1988).

1.10. Assessment of myeloperoxidase (MPO) activity in renal tissue after ischaemia/reperfusion in vivo: effects of antibodies against adhesion molecules used by PMNs

Myeloperoxidase, a characteristic constituent of PMNs (Bradley et al., 1982a; Schultz and Kaminker, 1962), has been used as a biochemical marker for the tissue content of PMNs. The established photometric assay of MPO employs o-dianisidine as the hydrogen donor for H_2O_2 (Bradley et al., 1982b). However, TMB may be used as a noncarcinogenic alternative hydrogen donor for the determination of peroxidase (Bos et al., 1981; Andrews and Krinsky, 1981; Holland et al., 1974) and can be shown to provide a more sensitive assay of human MPO than o-dianisidine (Suzuki et al., 1983). In an attempt to extend these findings, the sensitivities of TMB and o-dianisidine assays were directly compared in an assay of MPO derived from rat blood PMNs.
Polymorphonucleocyte (PMN) infiltration as assessed by tissue MPO assay has been shown in numerous tissues including the inflamed rat dermis (Bradley et al., 1982b), the ischaemic/reperfused dog and rabbit myocardium (Bednar et al., 1985; Mullane et al., 1985;

1987; Schierwagen et al., 1990), rat intestine (Grisham et al., 1986; Karasawa et al., 1991; Krawisz et al., 1984), rat liver (Augustin & Lutz, 1991) and dog gracilis muscle (Korthuis et al., 1988). A potential problem concerned with the assessment of MPO in renal tissue is that peroxidase activity may be effectively inhibited by renal tissue factors (Schierwagen et al., 1990; Hillegas et al., 1990; Ormrod et al., 1987). Schierwagen et al. (1990) have speculated that these factors may include H_2O_2 consuming systems. The aim of the present study was to employ measures that would overcome these peroxidase inhibitory factors. To this end, the recovery of exogenous horseradish peroxidase activity added to rat renal tissue was assessed as an index of inhibition. The validity of these measures was demonstrated by delineating a time course of renal PMN accumulation after 45min renal ischaemia and various periods of reperfusion *in vivo*. It was of further interest to investigate a role for adhesion molecules in renal PMN accumulation after ischaemia/reperfusion using recently available murine MoAbs against rat intercellular adhesion molecule-1 (ICAM-1) and rat CD18 of β_2-integrins (Tamatani & Miyasaki, 1990; Issekutz & Issekutz, 1992).

2. Methods

2.1. Rat model of ischaemia/reperfusion in vivo at Bath University

Male Wistar rats of Bath University Animal House stock weighing 250-300g maintained on standard laboratory chow and allowed water *ad libitum*, were anaesthetised with Hypnorm (fentanyl, 0.315mg/ml & fluanisone, 10mg/ml)/Hypnovel (midazolam, 5mg/ml) (3.3ml/kg i.p. of a solution comprising 1 pt Hypnorm, 1 pt Hypnovel, 2 pts sterile water) and placed on a warming pad at 37°C. Prolonged anaesthesia was afforded by the administration of Hypnorm (0.3ml/kg i.m.) every 30min following the initial dose of Hypnorm/Hypnovel (Flecknell, 1987). For a review of neuroleptanalgesia, see Green (1978). Following the administration of heparin (200U/kg via tail vein) in 0.9% (w/v) saline, the peritoneal cavity was opened by a midline abdominal incision and the renal arteries located by blunt dissection. Intestinal disturbance was kept to a minimum. Renal ischaemia was produced by left renal pedicle occlusion with a non-traumatic vascular clamp for 30min. The right sham-operated kidney served as a control. Reperfusion for 15min was elected by removal of the clamp.

2.2. Isolated perfused kidney technique

The cannulation of the renal arteries for renal perfusion *in vitro* was essentially as described by Nishiitsutso-Uwo et al. (1967), Schurek et al. (1975) and Linas et al. (1981). Briefly, the left renal artery was cannulated first with a Portex (OD 0.63mm) luer fitting catheter after ligation of the aorta between the origins of the right and left renal arteries. The right renal artery was cannulated via the superior mesenteric artery after ligation of the proximal aorta and adrenal artery. Kidneys were rapidly excised and perfused by a Watson-Marlow type MHRE 200 flow inducer in open circuit at 6ml/min with Krebs Henseleit solution (KHS) (pH 7.4) warmed to 37°C and gassed with 95% oxygen/ 5% carbon dioxide. The KHS was prefiltered through a pore size of 11μm to remove gross particulate matter. In early trials, in-line filters (0.45-0.8μm) soon became fouled and had to be discarded. The composition of KHS was (in mM): NaCl, 143; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 1.2; glucose, 11.7. Renal perfusion pressure was monitored with a Gould pressure transducer and displayed on a Gould 2200S chart recorder. Since flow rate was constant, changes in perfusion pressure were taken to reflect changes in renovascular resistance. Perfused kidneys were allowed to stabilise for 30min before interventions. Bolus doses were injected through the

kidney via an injection port in a volume which did not affect perfusion pressure (50μl).

2.3. Assessment of renal water content

Rat kidneys were first weighed wet, incubated at 60°C for 12h in an oven and then re-weighed to provide dry weight. The water content was assessed as the difference between wet and dry weights.

2.4. Renal vasodilation to histamine

Preparations were preconstricted with either methoxamine (3μM, Burton et al., 1990) or an equieffective concentration of potassium (30mM) and vasodilator responses (expressed as a percentage reduction in elevated perfusion pressure) to histamine (1-300nmoles), and papaverine (1-300nmoles) and acetylcholine (0.03-3 nmoles) as comparisons, were assessed.

2.5. Effects of NO synthase and cyclo-oxygenase inhibitors and histamine receptor antagonists on renal vasodilation to histamine

Two submaximal doses of histamine (10, 30nmoles) together with two doses of papaverine as internal controls (30nmoles papaverine equiactive with 30nmoles histamine and 100nmoles papaverine providing a maximal dilation) which gave reproducible responses with time, were subsequently employed to assess renal vasodilation to histamine and papaverine in potassium(30mM)- or methoxamine(3μM)-preconstricted preparations before and during perfusion with NO synthase or cyclo-oxygenase inhibitors or histamine receptor antagonists. In addition, a submaximal dose of acetylcholine (0.3nmoles eliciting 60% of E_{max}) was used to provide NO-dependent renal vasodilation in methoxamine(3μM)-preconstricted preparations. Vasodilator responses in these studies were expressed as a percentage of the maximal response to 100nmoles papaverine.

The pA_2 for ranitidine was estimated from a functional inhibition curve after Hill plot transformation, using dose-ratio analysis analogous to the Gaddum equation essentially as described by Lazareno & Birdsall (1993a;b).

2.6. Vasoconstriction to endothelins and effects of BQ123 and indoemethacin

An assessment of vasoconstrictor reactivity to endothelin-1 ET-1 (1-100pmoles), ET-2 (1-100pmoles) and ET-3 (1-100pmoles) was made in an attempt to profile the major ET/SX receptor subtype responsible. Three doses each of ET-1 and ET-3 (3,10,30pmoles) were subseqently chosen to assess vasoconstriction to ET-1 or ET-3 during perfusion with BQ123 (1μM, Ihara et al., 1992b; Télémaque et al., 1993) or indomethacin (10μM, Stier et al., 1992). Comparisons were made with separate time-matched control preparations. Any effect of interventions on non-specific vasoconstrictor reactivity was assessed by examining the vasoconstrictor response to a 10min perfusion with modified KHS containing 110mM KCl.

2.7. Renal vasoconstrictor reactivity <u>in vitro</u> after ischaemia <u>in vivo</u>

Vasoconstrictor reactivity to ET-1 (3-30pmoles), ET-3 (3-30pmoles), noradrenaline (1-300nmoles), 5-hydroxytryptamine (1-100 nmoles), and KCl (110 mM) was assessed in the isolated perfused kidney after 30min renal ischaemia or 30min normoperfusion as control, *in vivo*.

2.8. Isolation of rat blood PMNs

Blood collected from a carotid artery into heparin (10U/ml) from male CD Wistar rats anaesthetised with Sagatal (60mg/kg i.p.) was carefully layered onto Ficoll-Paque lymphocyte separation medium and spun at 300g for 30min. The lower ruddy layer was mixed with an equal volume of 1.8% (w/v) dextran in isotonic, pH7.4 phosphate buffered saline (PBS) and left to sediment for 40min. The resultant upper layer was spun at 200g for 10min and the pellet resuspended in first 5.8ml 0.21% (w/v) NaCl and then 0.5ml 1M KCl to lyse remaining erythrocytes. After washing in PBS, viable cells were enumerated in a Neubauer haemocytometer following dilution with 0.1% (w/v) trypan blue. Differential cell counts indicated the yield for PMNs to be routinely >90%. Samples were diluted in PBS to provide $1x10^6$ PMNs/ml.

2.9. Extraction of MPO from rat blood PMNs

Aliqouts of PMNs ($1x10^6$-$1x10^5$) were transferred to vials and the volume subsequently made up to 5ml with hexadecyltrimethylammonium bromide (HTAB) in 50mM pH6.0 phosphate buffer to give a final concentration of 0.5% (w/v). PMN suspensions were then disrupted for 30s using a Branson sonicator at 20% power and subsequently snap frozen in liquid nitrogen and thawed on three consecutive

occasions before a final 30s sonication. PMN suspensions were then incubated at 60°C for 2h (see below) to verify the heat resistance of MPO (Schierwagen et al., 1990). Incubated PMN suspensions were spun at 4000g for 12min and the MPO-rich supernate collected and stored at -22°C until assay.

2.10. Assay of MPO from rat blood PMNs

MPO was assayed photometrically in a 96-well plate incubated at 37°C using a Dynatech MR7000 plate reader controlled by a Toshiba 1000 microprocessor. The TMB assay of MPO was essentially as described by Suzuki et al. (1983). The assay mixture consisted of 20µl MPO-rich supernate, 10µl TMB (final concentration 1.6mM) dissolved in dimethylsulphoxide (DMSO) and 70µl H_2O_2 (final concentration 0.3mM) diluted in 80mM phosphate buffer pH5.4. The o-dianisidine assay of MPO was essentially as described by Bradley et al. (1982b). The assay mixture consisted of 20µl MPO-rich supernate and 80µl H_2O_2 (final concentration 0.147mM) diluted in 50mM phosphate buffer pH6.0 containing o-dianisidine dihydrochloride (final concentration 0.526mM). The reaction was initiated by the addition of H_2O_2 and the increase in absorbance was determined over 3min at 30s intervals at either 630nm (TMB) or 460nm (o-dianisidine).

2.11. Recovery of exogenous horseradish peroxidase activity from rat renal supernate

As an index of the inhibition of peroxidase activity by renal tissue, the recovery of exogenous horseradish peroxidase activity was assessed from renal supernate derived from non-ischaemic rat kidneys in a photometric assay using TMB. The activity of exogenous horseradish peroxidase in the presence of 0.5% (w/v) HTAB was defined as 100% recovery. In an attempt to enhance the recovery of exogenous horseradish peroxidase activity from renal supernate, the renal supernate was heat-inactivated by incubating at 60°C for 2h in a water bath (Schierwagen et al., 1990) and/or the assay H_2O_2 concentration was raised 10-fold. The assay mixture consisted of 10μl TMB (final concentration 1.6mM) dissolved in DMSO, 50μl H_2O_2 (final concentration 0.3 or 3.0mM) diluted in 80mM phosphate buffer pH5.4, 20μl heat-inactivated or untreated renal supernate or 20μl 0.5% (w/v) HTAB (as control) and 20μl horseradish peroxidase diluted as for H_2O_2 (final concentration 8.7-43.3μg/l). Horseradish peroxidase activity was expressed as the initial rate of change in absorbance (δA/min) per unit concentration of enzyme (1mg/l).

2.12. Determination of molar absorption coefficient for TMB-dehydrogenation product

TMB present over a range of initial concentrations was dehydrogenated by H_2O_2 in the presence of excess horseradish peroxidase activity and the peak absorbance increase determined at 630nm. The assay mixture consisted of 10μl TMB (final concentration 0.2-1.6mM) dissolved in DMSO, 70μl H_2O_2 (final concentration 0.04-0.3mM) and 20μl horseradish peroxidase (final concentration 41.6mg/l) diluted in 80mM phosphate buffer, pH5.4. The ratio of TMB:H_2O_2 was maintained at 5.3:1 to avoid the oxidation of TMB-dehydrogenation product by excess H_2O_2.

2.13. Rat model of renal ischaemia/reperfusion <u>in vivo</u> at Roche Research Centre

Male CD Wistar rats (300-400g) were anaesthetised with Sagatal (60mg/kg i.p.) and the body temperature maintained at 37°C by a warming pad. Both kidneys were exposed via flank incisions and kept moist with warmed 0.9% (w/v) saline. After the administration of heparin (200U/kg via tail vein), the left renal pedicle was occluded by an atraumatic vascular clamp for 45min. Reperfusion was elected for 0.5-6h by removal of the clamp and visually confirmed before postischaemic and sham-operated kidneys were restored to the body cavity

and the wounds sutured. Temgesic (0.5mg/kg s.c.) was administered upon reperfusion to provide post-operative analgesia. Renal reperfusion was terminated by cervical dislocation of the animal. Kidneys were then excised, blotted dry and weighed. Kidneys were subsequently immersed in 0.5% (w/v) HTAB in 50mM phosphate buffer pH6.0 and stored at -22°C until MPO extraction and assay.

2.14. Renal MPO extraction

Frozen rat kidneys were thawed at room temperature and macerated with an Ultra-Turrax T25 blender for 30s. Sample volumes were then made up to 10ml with 0.5% (w/v) HTAB before disruption and freeze-fractionation as described for the extraction of MPO from rat blood PMNs. Samples were incubated at 60°C for 2h in a water bath and then spun at 4000g for 12min. The supernate was collected for MPO assay.

2.15. Renal MPO assay

Renal MPO activity was assessed photometrically at 630nm as described for the assessment of rat blood PMN MPO activity using TMB as substrate. The assay mixture consisted of 20μl heat-inactivated renal supernate, 10μl TMB (final concentration 1.6mM) dissolved in DMSO and

70μl H_2O_2 (final concentration 3.0mM) diluted in 80mM phosphate buffer pH5.4. Renal MPO activity was expressed in units (U) where 1U represents that amount of enzyme degrading 1μmole H_2O_2/min under the stated conditions. MPO activity was standardised with respect to the wet weight of the sham-operated kidney.

2.16. Effect of monoclonal antibodies (MoAbs) against adhesion molecules used by PMNs on renal MPO activity

Murine anti-rat ICAM-1 or murine anti-rat CD18 MoAb was administered to animals both 5min before 45min renal ischaemia (20μg/kg via tail vein) and at the commencement of 1h reperfusion (20μg/kg via tail vein) *in vivo*. The isotype-matched antibody murine IgG_1 or an unrelated murine antibody, IgG_{2a}, was also administered in an identical manner to serve as controls. Vehicle control animals received 0.9% (w/v) saline (0.5ml/kg via tail vein) at the appropriate time points. Sham-operated contralateral kidneys provided control preparations for ischaemic/reperfused kidneys.

2.17. Effect of murine IgG_1 on recovery of exogenous horseradish peroxidase activity from rat renal supernate

In order to examine the possibility that murine antibodies might directly affect tissue peroxidase activity, the recovery of exogenous horseradish peroxidase activity was assessed from renal supernate derived from non-ischaemic kidneys exposed to murine IgG_1 (40μg/kg i.v. total) *in vivo*. The activity of horseradish peroxidase in the presence of 0.5% (w/v) HTAB was defined as 100% recovery.

2.18. Fluorescence activated cell sorting (FACS) analysis

Aliquots of rat blood PMNs ($0.5x10^6$ cells) were suspended in 50μl PBS/0.2% (w/v) sodium azide together with 4μg of anti-ICAM-1 or anti-CD18 MoAb or murine IgG_1 or IgG_{2a} antibody and incubated at -4°C for 30min. Cell suspensions were then washed twice in 1ml PBS/0.2% (w/v) sodium azide and resuspended in 50μl PBS/0.2% (w/v) sodium azide together with 0.5μg fluorescently labelled bovine anti-murine IgG R-phycoerythrin conjugate $F(ab')_2$ fragment and incubated at -4°C for 30min. Cells were then washed twice and finally resuspended in PBS/0.2% (w/v) sodium azide and stored at -4°C until FACS analysis using a Becton Dickenson FACSar.

2.19. Pharmacological agents and solutions

Agents used were noradrenaline bitartrate, acetylcholine hydrochloride, histamine hydrochloride, mepyramine maleate, ranitidine hydrochloride, 5-hydroxytryptamine creatinine sulphate, verapamil hydrochloride, N^G-nitro-L-arginine methyl ester, N^G-nitro-L-arginine, heparin sodium, ascorbic acid and indomethacin (Sigma), thioperamide hydrochloride (Research Biochemicals Inc.), endothelins-1, -2 -3 (Novabiochem), BQ123 (Peninsula), murine anti-rat-ICAM-1 and murine anti-rat-LFA-1 ß chain (anti-CD18) MoAbs (British Biotechnology), murine IgG_1, murine IgG_{2a} and bovine anti-mouse IgG R-phycoerythrin conjugate $F(ab')_2$ fragment (Sigma Immunochemicals), 3,3',5,5'-tetramethylbenzidine, o-dianisidine hydrochloride, Type II horseradish peroxidase, dimethylsulphoxide, sodium azide and hexadecyltrimethylammonium bromide (Sigma), Giemsa stain, trypan blue, hydrogen peroxide, dextran (250KDa), sodium di-hydrogen phosphate monohydrate, di-sodium hydrogen phosphate hexahydrate (British Drug House), sterile 0.9% (w/v) saline (Fresenius), Temgesic (buprenorphine hydrochloride) (Reckitt and Colman), Sagatal (pentobarbitone sodium) (May and Baker) and Ficoll-Paque (Pharmacia). Indomethacin was dissolved with an equal amount of di-sodium carbonate in 0.9% (w/v) saline. All other agents were dissolved in 0.9% (w/v) saline and agents to be perfused were diluted to the working

concentration in KHS. Solutions of noradrenaline were made up in 0.9% (w/v) saline containing 0.1mM ascorbate. Modified KHS containing 30 or 110mM KCl was prepared by including the desired quantity of KCl and osmotically adjusting by proportionately reducing the content of NaCl.

2.20. Statistics

The comparison of two group means was conducted using Student's t-test (Matthews & Farewell, 1988). The comparison of multiple group means was made using 1 way analysis of variance (1W-ANOVA) while the comparison of repeated measures was conducted using 2W-ANOVA (Bailey, 1981; Snedecor & Cochran, 1980). The comparison of a control group mean with each of a number of treatment group means was conducted using Dunnett's test (Tallarida & Murray, 1981; Wallenstein, 1980). Curve fitting was conducted according to the method of least squares and preceded linear regression analysis (Goldstein, 1964). The comparison of slopes of linear regression lines was conducted using Student's t-test for parallelism (Tallarida & Murray, 1981). Values are mean ± standard error of the mean (SEM). Statistical significance was accepted at $P<0.05$.

3. Results

3.1. Renal vasodilation to histamine and papaverine

When rat kidneys were perfused at 6ml/min, basal renal perfusion pressure was in the range 70-90 mmHg. Sustained elevations in perfusion pressure obtained with either potassium (30mM) or methoxamine (3μM, Burton et al., 1990) were in the range 135-220 mmHg. Preconstriction with methoxamine (3μM) was often unstable (Figure 2). Preconstriction with phenylephrine (0.5μM, Oyekan et al., 1991), noradrenaline (0.5μM, Bhardwaj & Moore, 1988), angiotensin II (100nM, Scholz & Kurtz, 1990) and endothelin-1 (3-100 pmoles, Baydoun et al., 1989) was also found to be unstable in pilot studies of 1-2 preparations only.

Histamine (1-300nmoles) (Figure 3) and papaverine (1-300nmoles) (Figure 4) elicited dose-dependent reductions in perfusion pressure elevated to the same level with either potassium (30mM) or methoxamine (3μM) (Figure 5). The ED_{50} for histamine against potassium-induced tone (7.5±1.0nmoles, n=20) was not significantly different from the ED_{50} for histamine against methoxamine-induced tone (9.7±2.1nmoles, n=8). However, vasodilator responses to histamine (1-300nmoles) were significantly depressed in potassium(30mM)-preconstricted preparations when

Figure 2. Representative trace showing aberrant vasoconstrictor responsiveness to methoxamine (3μM) in the isolated perfused rat kidney.

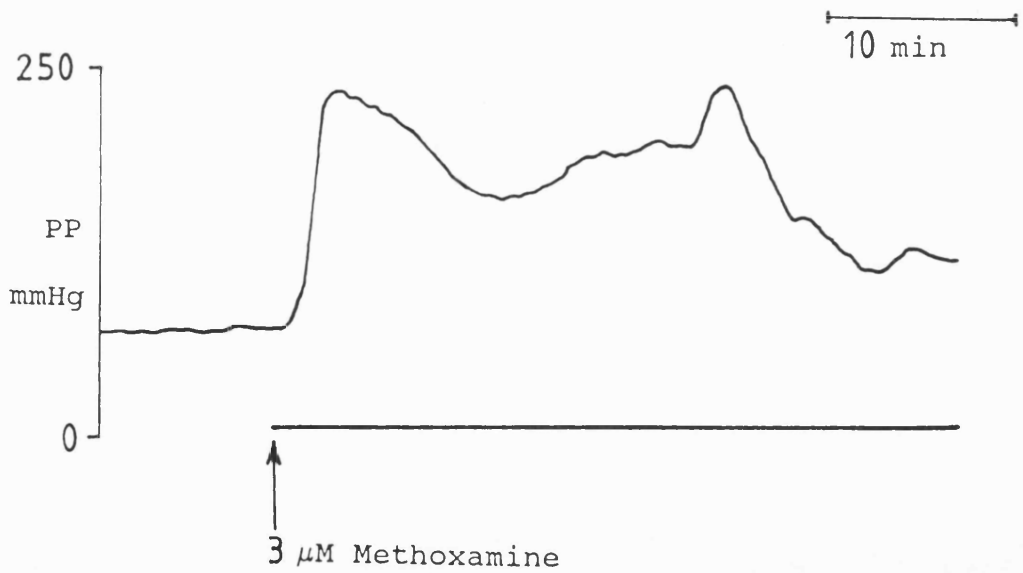

10 min
250
PP
mmHg
0
3 μM Methoxamine

Figure 3. Vasodilation to histamine (HA) in the isolated perfused rat kidney preconstricted with methoxamine (3μM) (n=8) (open circles) or potassium (30mM) (n=20) (closed circles). Methoxamine (3μM) and potassium (30mM) elevated perfusion pressure (PP) to 173±9 (n=8) and 166±6 (n=20) mmHg, respectively. Decreases in PP are expressed as a percentage reduction in elevated PP. ★P<0.01 with respect to vasodilator responses in methoxamine(3μM)-preconstricted preparations. Values are mean±SEM.

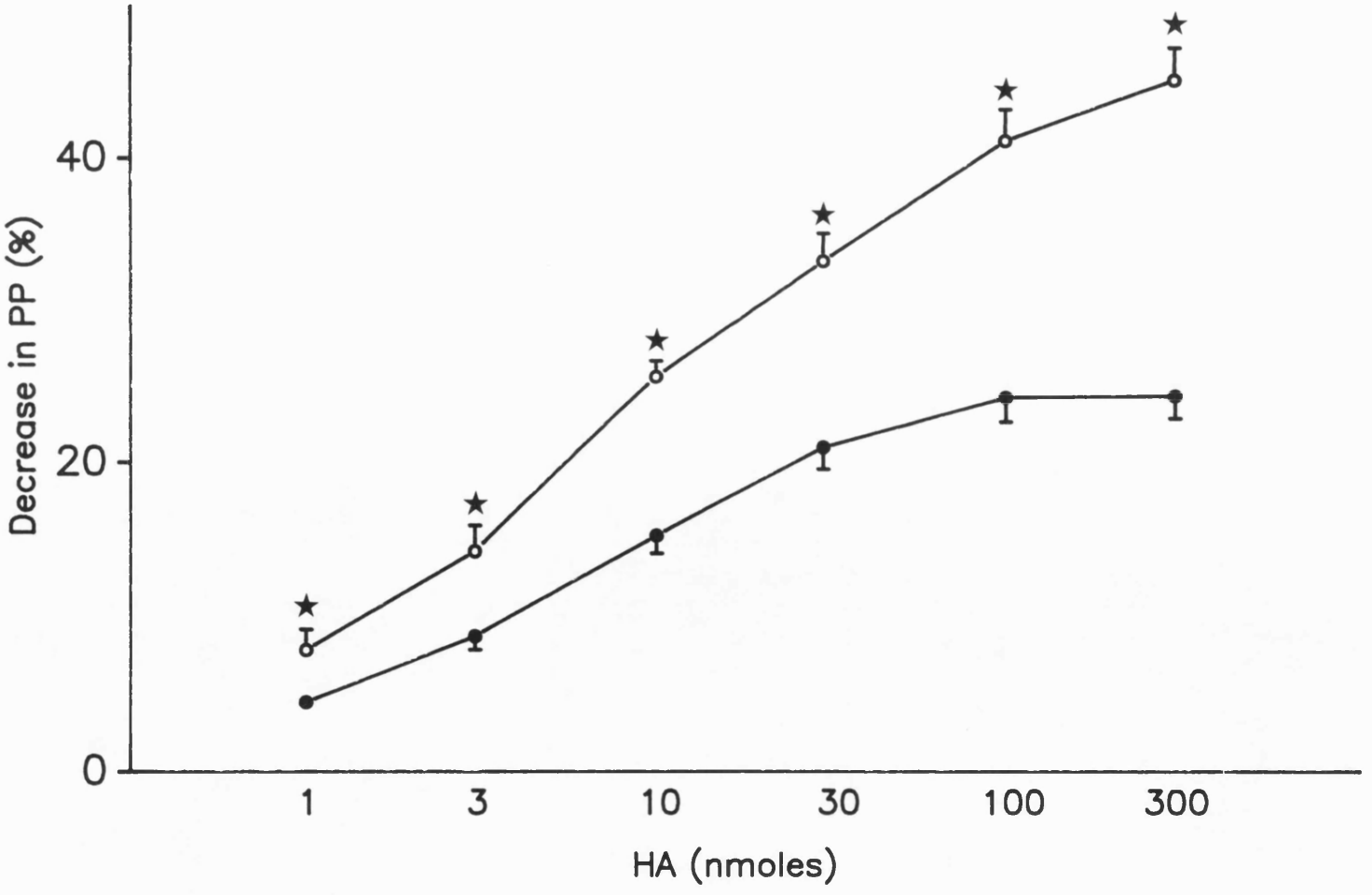

Decrease in PP (%)
40
20
0
1
3
10
30
100
300
HA (nmoles)

Figure 4. Vasodilation to papaverine (PAP) in the isolated perfused rat kidney preconstricted with methoxamine (3μM) (n=5) (open circles) or potassium (30mM) (n=14) (closed circles). Methoxamine (3μM) and potassium (30mM) elevated perfusion pressure (PP) to 157±9 (n=5) and 175±9 (n=14) mmHg, respectively. Decreases in PP are expressed as a percentage reduction in elevated PP. Values are mean±SEM.

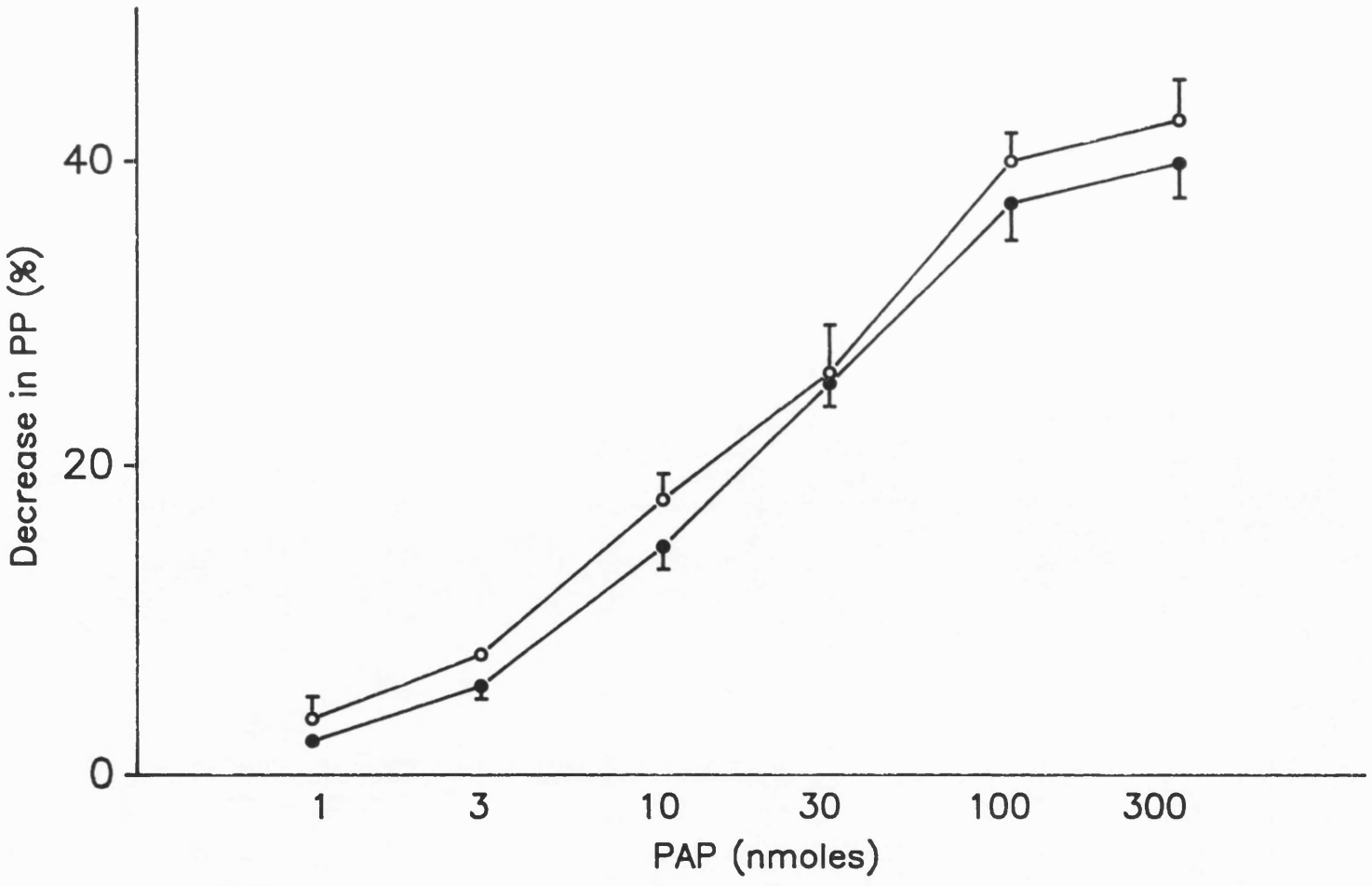
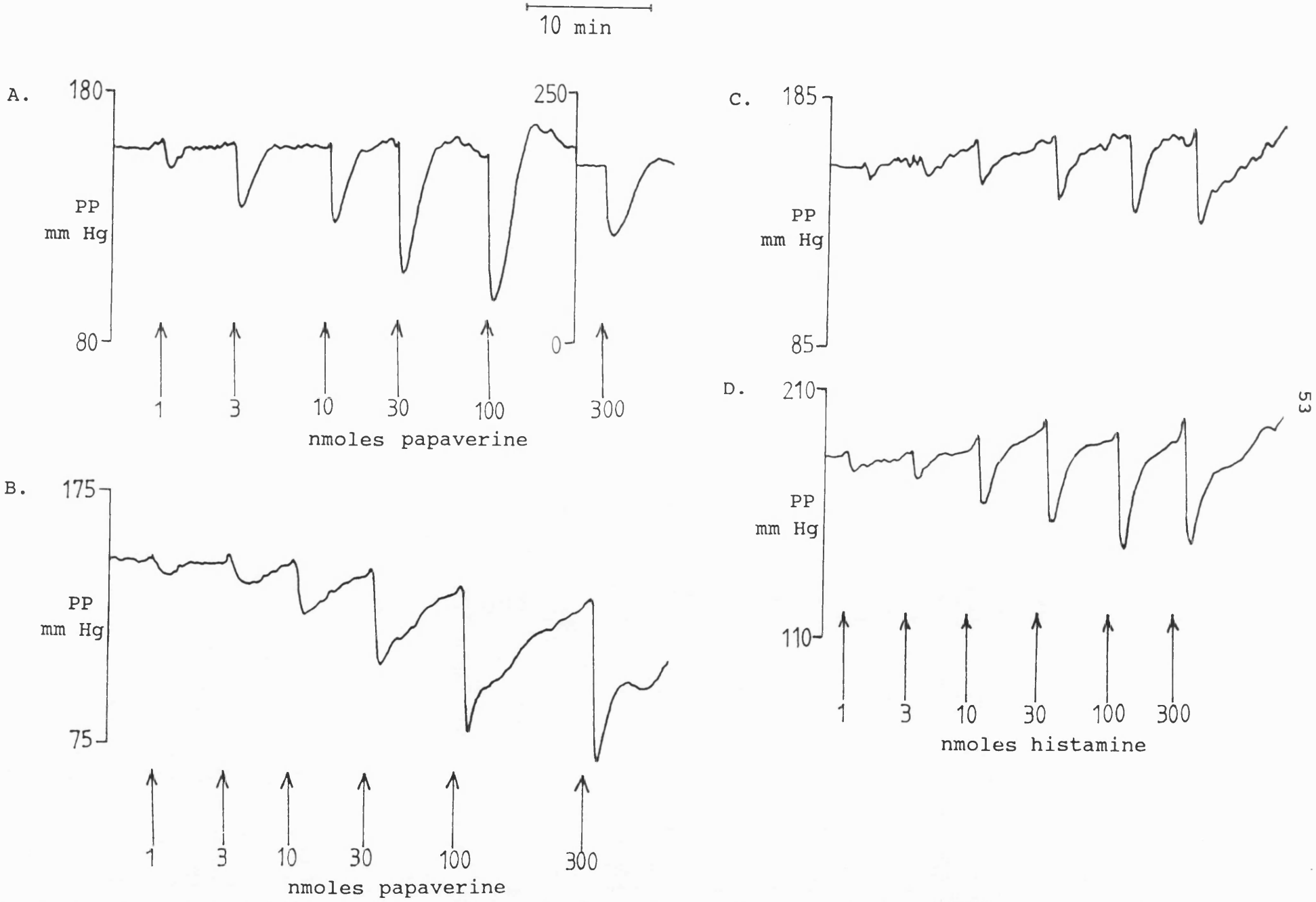

40
20
0
Decrease in PP (%)
1
3
10
30
100
300
PAP (nmoles)

Figure 5. Experimental trace showing vasodilation to histamine (1-300nmoles) and papaverine (1-300nmoles) in the isolated perfused rat kidney preconstricted with potassium (30mM) (A & C) or methoxamine (3μM) (B & D).


10 min
A.
180
250
PP
mm Hg
80
0
1
3
10
30
100
300
nmoles papaverine
B.
175
PP
mm Hg
75
1
3
10
30
100
300
nmoles papaverine
C.
185
PP
mm Hg
85
D.
210
PP
mm Hg
110
1
3
10
30
100
300
nmoles histamine

compared to methoxamine(3μM)-preconstriction (Figure 3). In contrast, vasodilator responses to papaverine were identical in potassium-preconstricted (ED_{50}=21.5±2.5 nmoles, n=14; E_{max}=40±2%, n=14) and methoxamine-preconstricted (ED_{50}=20.3±4.6nmoles, n=5; E_{max}=40±2%, n=5) preparations. Acetylcholine (0.03-3nmoles) elicited dose-dependent reductions in perfusion pressure elevated with methoxamine (3μM) (ED_{50}=0.15±0.02nmoles, n=5; E_{max}=24±2%, n=5) (Figure 6). Vasodilator responses to acetylcholine (0.03-3nmoles) were poorly graded and significantly depressed in potassium-preconstricted preparations (n=15) (Figure 6).

3.2. Effects of histamine receptor antagonists on renal vasodilation to histamine

Ranitidine (0.1-10μM) significantly depressed vasodilation to 30nmoles histamine in a concentration-dependent manner and effectively abolished the response at 10μM (97% inhibition, n=7) (Figure 7). The pA_2 for ranitidine was estimated to be 6.67±0.09 (n=7) from the functional inhibition curve. Vasodilator responses to 30nmoles papaverine (equiactive with 30nmoles histamine) (Figure 7) and 100nmoles papaverine were not diminished in the presence of ranitidine (0.1-10μM).

Figure 6. Vasodilation to acetylcholine (ACh) in the isolated perfused rat kidney preconstricted with methoxamine (3μM) (n=5) (open circles) or potassium (30mM) (n=15) (closed circles). Methoxamine (3μM) and potassium (30mM) elevated perfusion pressure (PP) to 177±7 (n=5) and 162±5 (n=15) mmHg, respectively. Decreases in PP are expressed as a percentage reduction in elevated PP. ★P<0.01 with respect to vasodilator responses in methoxamine(3μM)-preconstricted preparations. Values are mean±SEM.

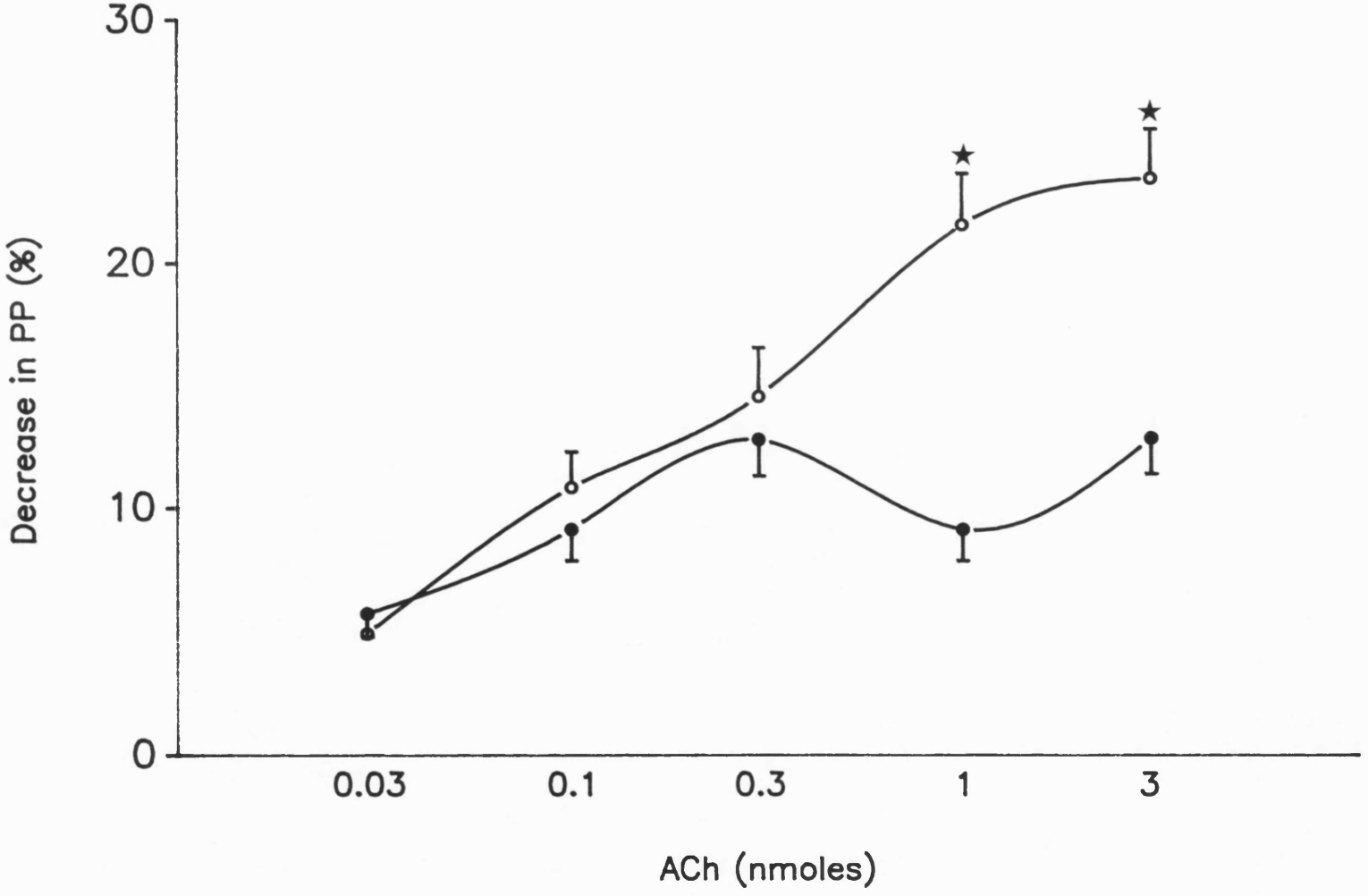
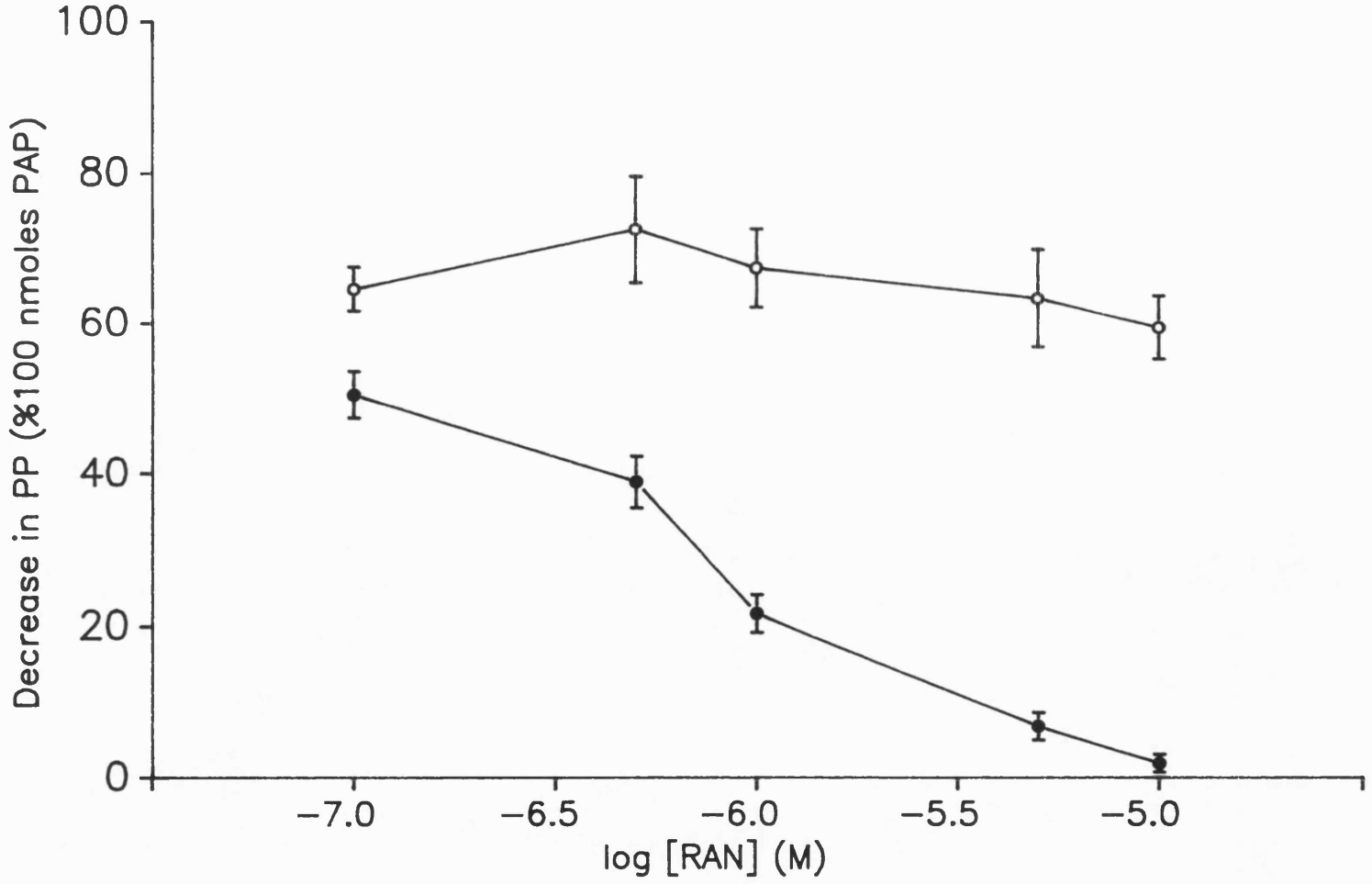

30
20
10
0
Decrease in PP (%)
0.03
0.1
0.3
1
3
ACh (nmoles)

Figure 7. Functional inhibition curve featuring vasodilation to histamine (HA) (30nmoles) (filled circles) and papaverine (PAP) (30nmoles) (open circles) in the potassium(30mM)-preconstricted isolated perfused rat kidney in the presence of graded concentrations of ranitidine (0.1-10μM) (n-7). Decreases in perfusion pressure (PP) are expressed as a percentage of the maximal decrease in PP obtained with 100nmoles PAP. Values are mean±SEM.

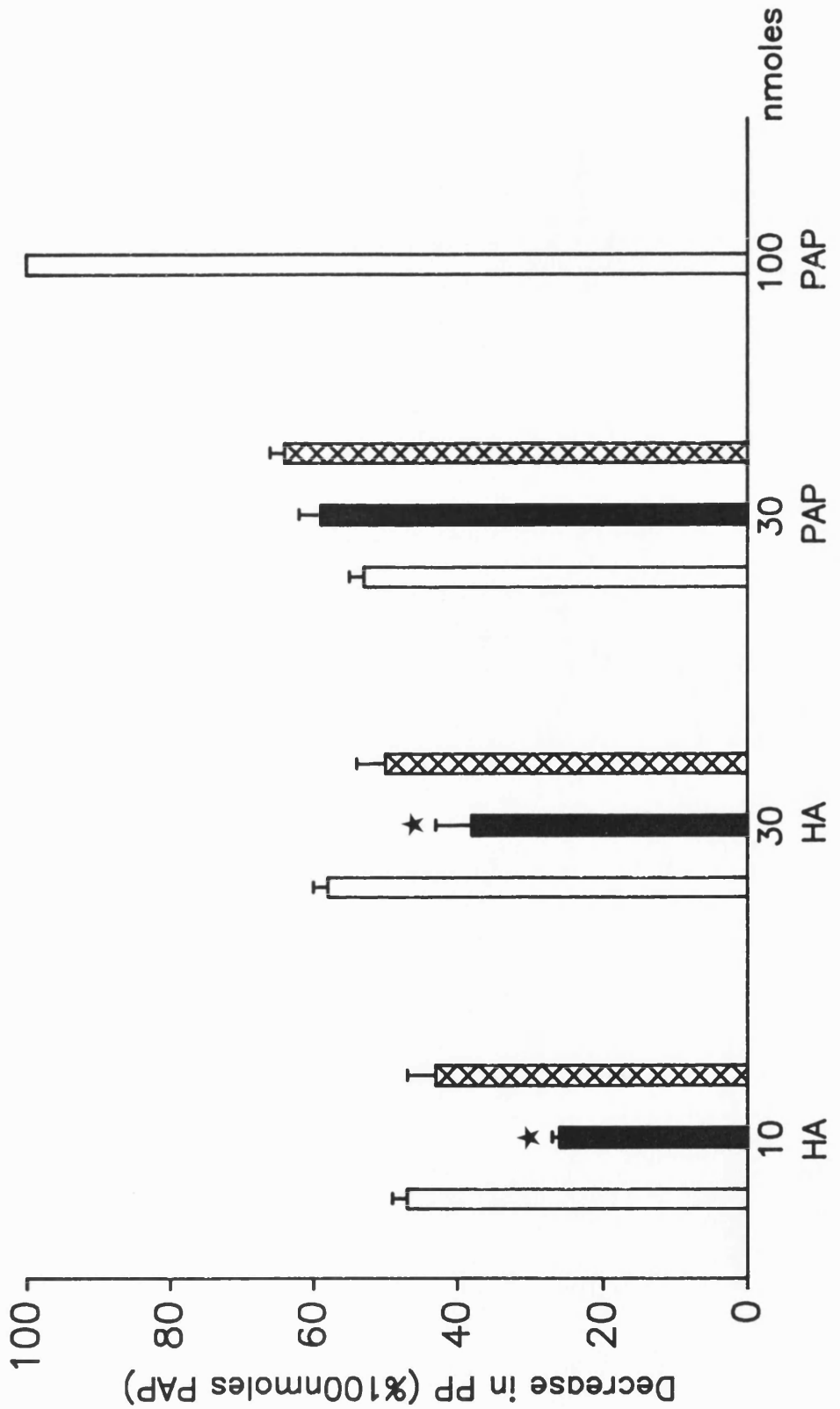

100
80
60
40
20
0
Decrease in PP (%100 nmoles PAP)
−7.0
−6.5
−6.0
−5.5
−5.0
log [RAN] (M)

Mepyramine at 1μM, a concentration presumed to produce effective H_1 receptor blockade (Hill & Young, 1980; Van de Voorde & Leusen, 1983b), significantly depressed but did not abolish, vasodilation to histamine (10, 30nmoles) (Figure 8). Vasodilation to 30nmoles papaverine equiactive with 30nmoles histamine was unaffected by this concentration of mepyramine. Thioperamide at 10μM did not significantly affect vasodilation to histamine (10, 30nmoles) or papaverine (30nmoles) (Figure 8). This concentration of thioperamide has been shown to fully reverse the histamine-mediated presynaptic inhibition of depolarisation-induced histamine release from rat brain slices (Arrang et al., 1987).

3.3. Effects of L-NAME, L-NOARG and indomethacin on elevated renal perfusion pressure

Perfusion pressure in potassium(30mM)-preconstricted preparations before and after L-NAME (0.3mM) was 186±9 and 193±9mmHg, respectively ($P>0.05$, n=5). Perfusion pressure before and after L-NAME (0.3mM) in methoxamine(3μM)-preconstricted preparations was 192±4 and 187±4mmHg, respectively ($P>0.05$, n=6). In contrast to the inefficacy of L-NAME (0.3mM), L-NOARG (0.3mM) raised perfusion pressure by 62±12 mmHg from 145±8 to 207±8 mmHg ($P<0.01$, n=6) in potassium(30mM)-preconstricted preparations. Indomethacin (10μM, Stier et al., 1992)

Figure 8. Vasodilation to histamine (HA) (10, 30nmoles) and papaverine (PAP) (30nmoles) in the potassium(30mM)-preconstricted isolated perfused rat kidney before (n=11) (open bars) and during perfusion with mepyramine (1μM) (closed bars) (n=6) or thioperamide (10μM) (cross-hatched bars) (n=5). Decreases in perfusion pressure (PP) are expressed as a percentage of the maximal decrease in PP obtained with 100nmoles PAP. ★P<0.05 with respect to before treatment. Values are mean±SEM.


Decrease in PP (%100nmoles PAP)
100
80
60
40
20
0
10
HA
30
HA
30
PAP
100
PAP
nmoles

significantly decreased perfusion pressure by 18±5mmHg from 145±4 to 127±4mmHg ($P<0.01$, n=6) in potassium(30mM)-preconstricted preparations.

3.4. Effects of L-NAME, L-NOARG and indomethacin on renal vasodilation to histamine

Vasodilation to histamine (10, 30nmoles) was unaffected by L-NAME (0.3mM), L-NOARG (0.3mM) or indomethacin (10μM) in potassium(30mM)-preconstricted preparations (Figure 9) and furthermore was unaffected by L-NAME (0.3mM) in methoxamine(3μM)-preconstricted preparations (Figure 10). The vasodilator response to a submaximal dose of acetylcholine (0.3nmoles eliciting 60% of E_{max}) was however significantly diminished by L-NAME (0.3mM) in methoxamine(3μM)-preconstricted preparations (Figure 10). The vasodilator response to acetylcholine (0.3nmoles) was unchanged in the time-matched control group (23±2% vs 25±2% of a 100nmoles papaverine response, $P>0.05$, n=6).

3.5. Renal vasoconstriction to ET-1, ET-2 and ET-3

ET-1, ET-2 and ET-3 elicited similar, dose-dependent increases in perfusion pressure over the same dose range (1-100 pmoles) (Figure 11). The E_{max} for ET-1, ET-2 and ET-3 was 153±11 (n=6), 159±12 (n=4) and 167±9 mm Hg (n=5)

Figure 9. Vasodilation to histamine (HA) (10, 30nmoles) and papaverine (PAP) (30nmoles) in the potassium(30mM)-preconstricted isolated perfused rat kidney before (open bars) (n=17) and during perfusion with L-NAME (0.3mM) (closed bars) (n=6), L-NOARG (cross-hatched bars) (n=6) or indomethacin (10μM) (diagonal bars) (n=6). Decreases in perfusion pressure (PP) are expressed as a percentage of the maximal decrease in PP obtained with 100nmoles PAP. Values are mean±SEM.

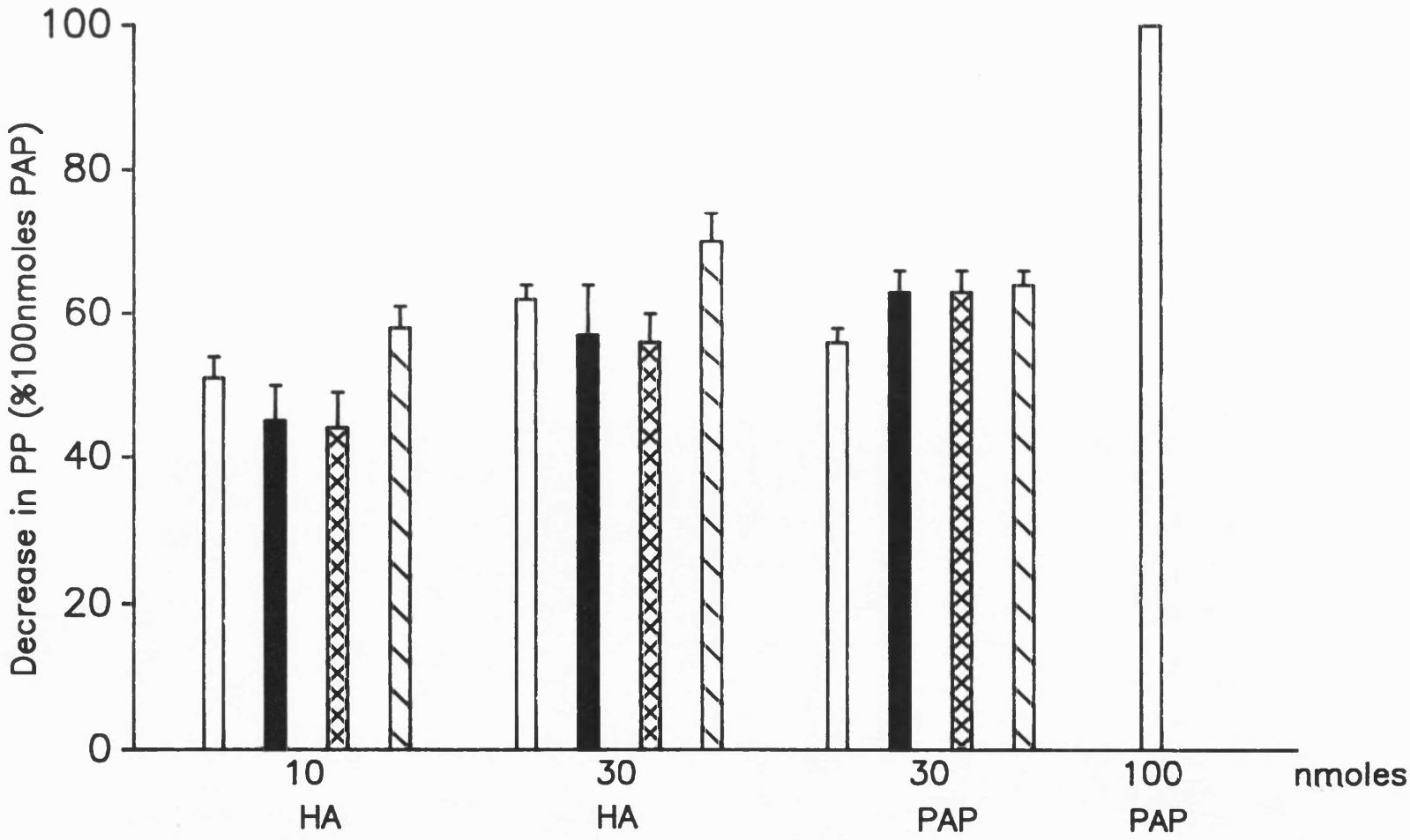

Decrease in PP (%100nmoles PAP)
100
80
60
40
20
0
10
HA
30
HA
30
PAP
100
PAP
nmoles

Figure 10. Vasodilation to acetylcholine (ACh) (0.3nmoles), histamine (HA) (10, 30nmoles) and papaverine (PAP) (30nmoles) in the methoxamine(3μM)-preconstricted isolated perfused rat kidney before (open bars) (n=6) and during perfusion with L-NAME (0.3mM) (closed bars) (n=6). Decreases in perfusion pressure (PP) are expressed as a percentage of the maximal decrease in PP obtained with 100nmoles PAP. ★P<0.01 with respect to vasodilator response to ACh (0.3nmoles) before treatment. Values are mean±SEM.

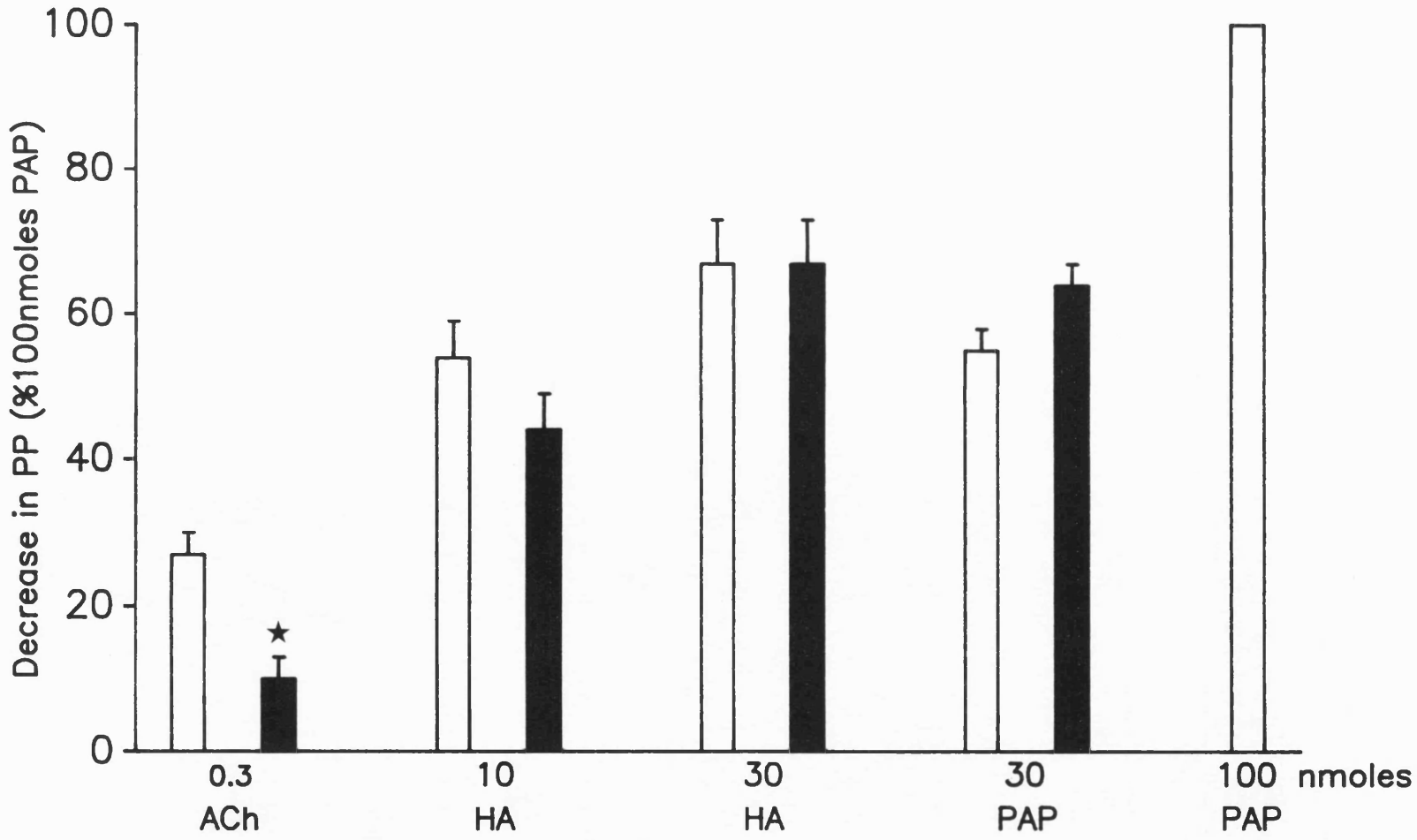
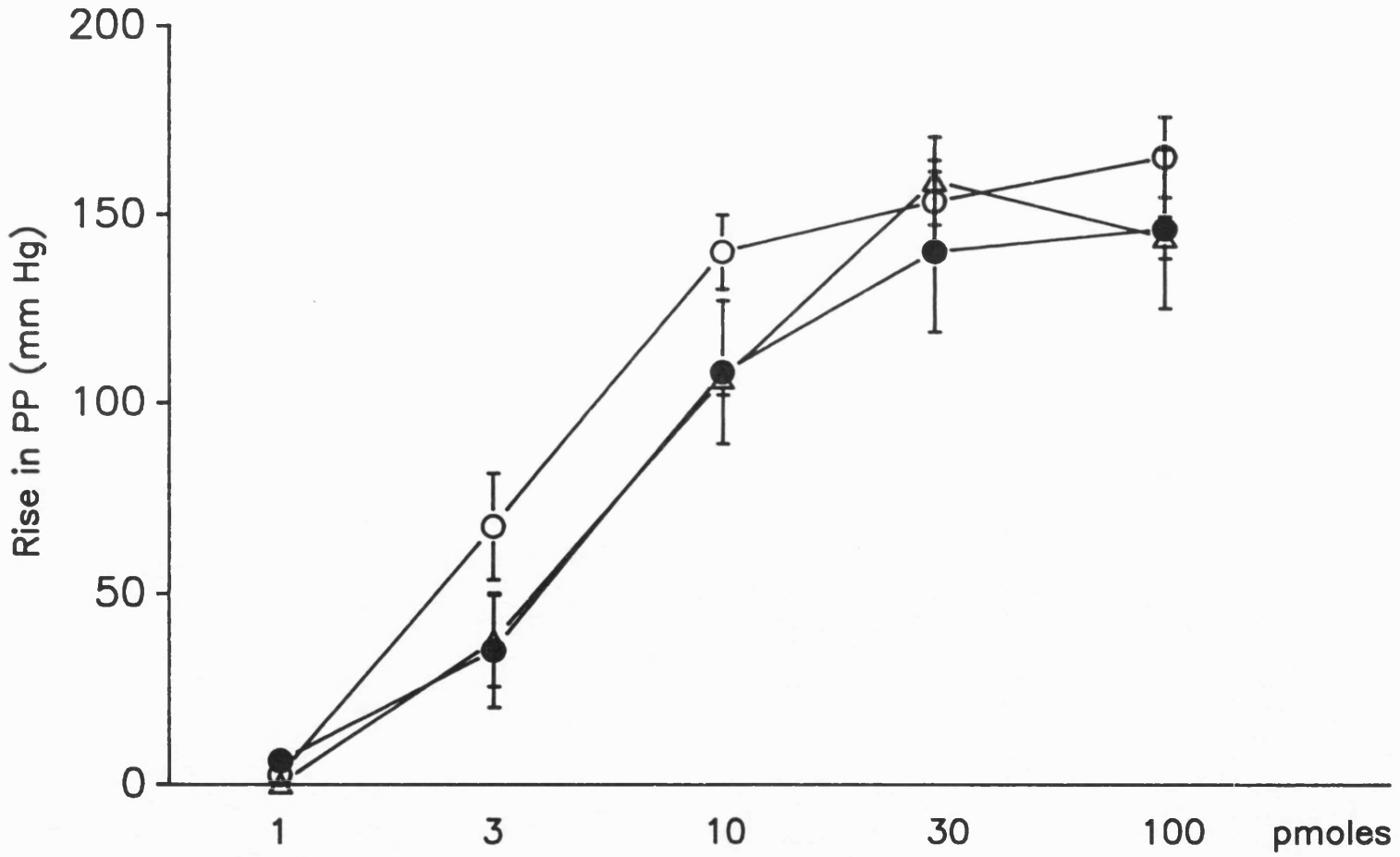

Decrease in PP (%100nmoles PAP)
100
80
60
40
20
0
★
0.3
ACh
10
HA
30
HA
30
PAP
100
PAP
nmoles

Figure 11. Vasoconstriction to endothelin-1 (ET-1) (open circles) (n=6), ET-2 (triangles) (n=4) and ET-3 (closed circles) (n=5) in the isolated perfused rat kidney. Basal perfusion pressure (PP) was in the range 70-81 mmHg. Values are mean±SEM.


200
150
100
50
0
Rise in PP (mm Hg)
1
3
10
30
100
pmoles

(P>0.05). Elevations in perfusion pressure due to ETs were transient. The time to 75% recovery from comparable peak rises in perfusion pressure due to ETs was significantly abbreviated for 30pmoles ET-3 relative to 30pmoles ET-1 and ET-2 (Figures 12 & 13).

3.6. Effect of BQ123 on renal vasoconstriction to ET-1 and ET-3

BQ123(1μM, Télémaque et al., 1993; Ihara et al., 1992b) showed no effect on either basal perfusion pressure or the non-specific vasoconstriction to KCl(110mM). Basal perfusion pressure in the presence and absence of BQ123(1μM) was 70±3 (n=6) and 79±8 mmHg (n=6), respectively (P>0.05). KCl(110mM) raised perfusion pressure by 116±3 (n=6) and 112±8 mmHg (n=6) in the presence and absence of BQ123(1μM), respectively (P>0.05). Peak vasoconstriction to the lower doses of ET-1 examined was significantly depressed by BQ123(1μM) (Figure 14). In addition, the time to 75% recovery from the peak rise in perfusion pressure due to 30pmoles ET-1 was significantly attenuated in the presence of ET_A receptor blockade (Figures 15 & 16). Indeed, the time to 75% recovery from peak vasoconstriction to 30pmoles ET-1 and 30pmoles ET-3 was similar in the presence of BQ123 (1μM). Peak rises in perfusion pressure due to 30pmoles ET-1 and ET-3 in the presence and absence of BQ123(1μM)

Figure 12. The time to 75% recovery from peak vasoconstrictor responses to endothelin-1 (ET-1) (open bars) (n=6), ET-2 (closed bars) (n=4) and ET-3 (cross-hatched bars) (n=5) in the isolated perfused rat kidney.

★ $P<0.01$ with respect to ET-1 and ET-2. Values are mean±SEM.

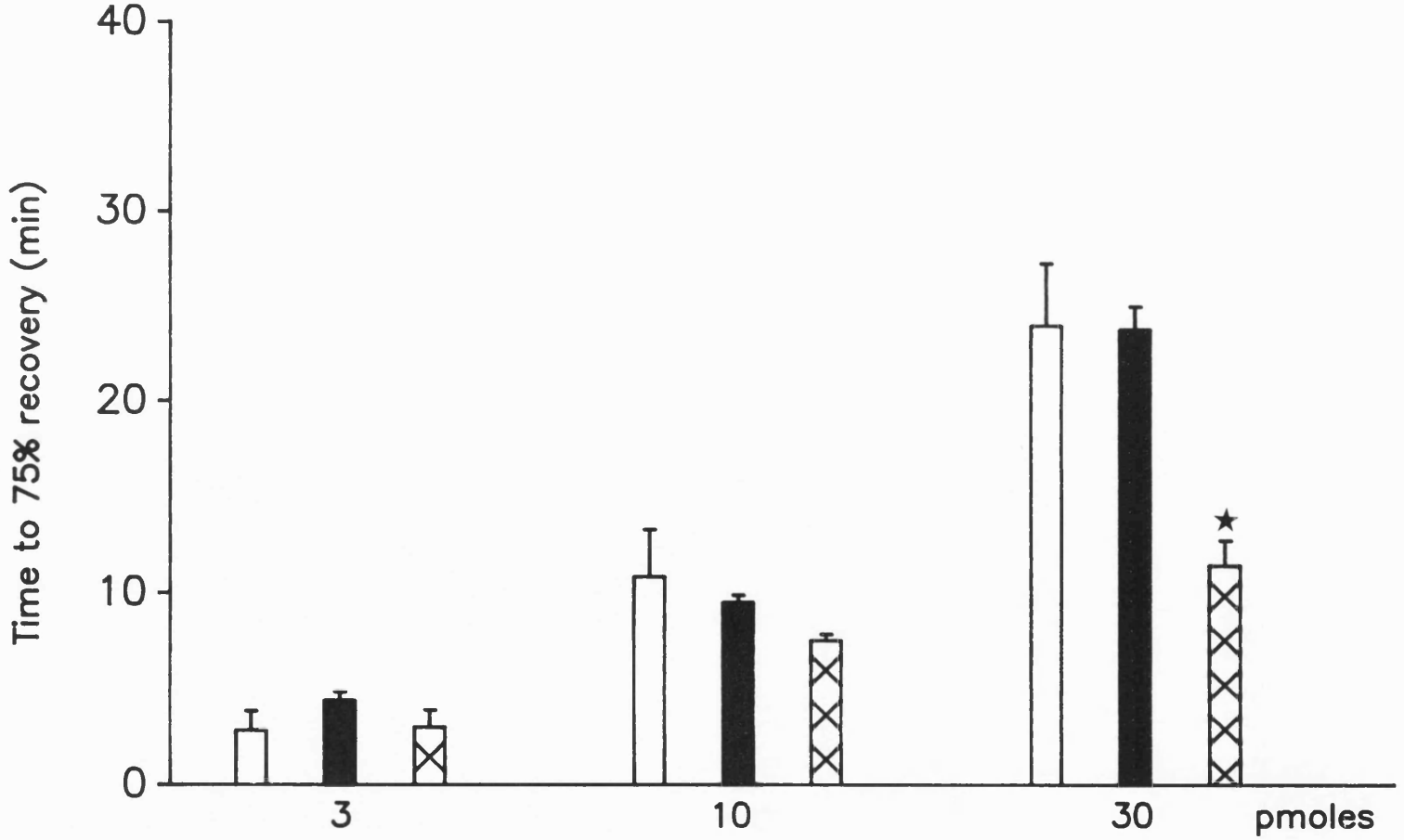

Time to 75% recovery (min)
40
30
20
10
0
3
10
30
pmoles
★

Figure 13. Experimental trace showing rises in perfusion pressure (PP) due to (A) endothelin-1 (ET-1), (B) ET-2 and (C) ET-3 (all 3-30pmoles) in the isolated perfused rat kidney.

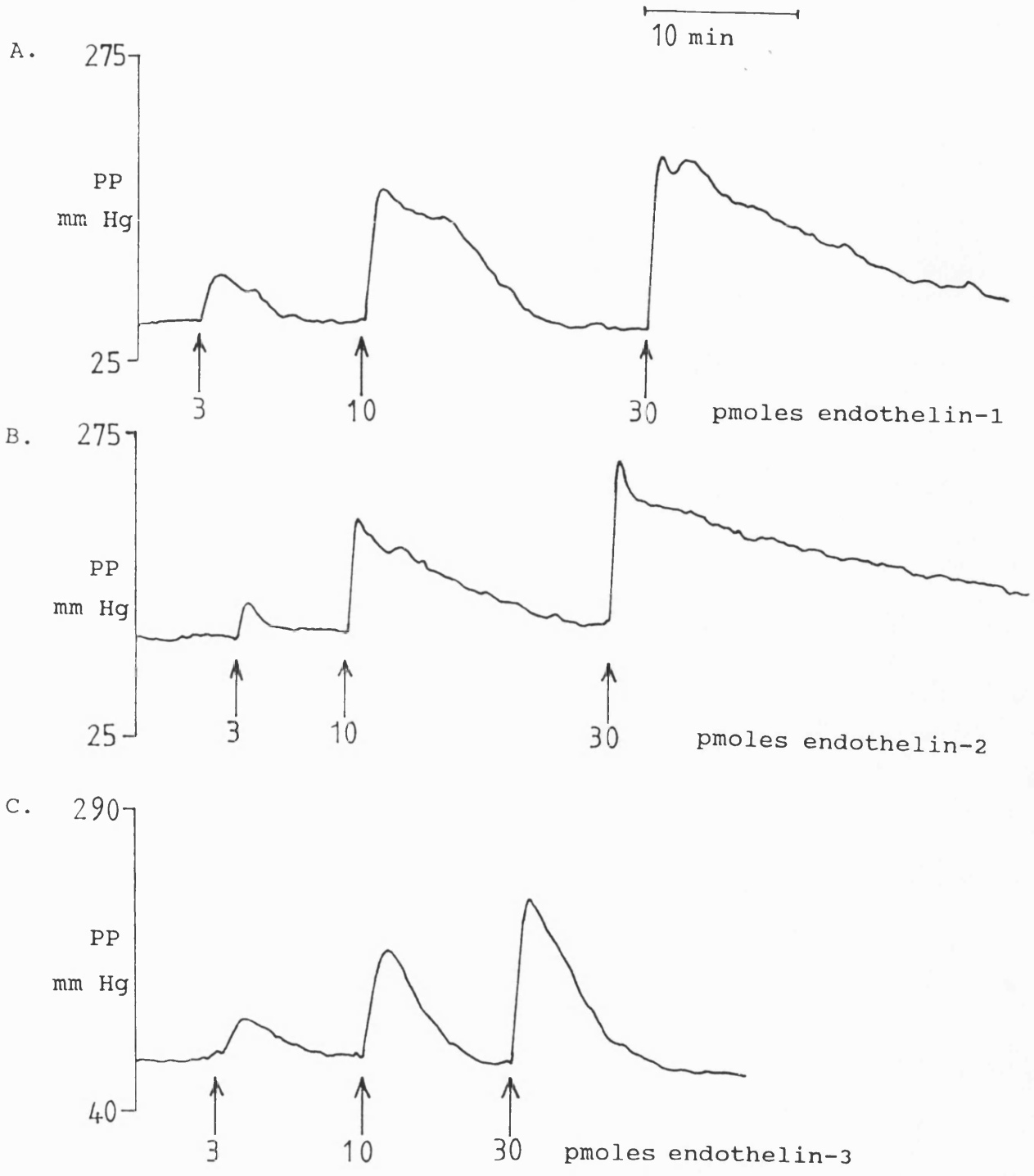

10 min
A.
275
PP
mm Hg
25
3
10
30
pmoles endothelin-1
B.
275
PP
mm Hg
25
3
10
30
pmoles endothelin-2
C.
290
PP
mm Hg
40
3
10
30
pmoles endothelin-3

Figure 14. Rises in perfusion pressure (PP) to endothelin-1 (ET-1) in the isolated perfused rat kidney in the absence (open bars) (n=6) or presence (closed bars) (n=6) of BQ123 (1μM). ★ $P<0.05$ with respect to the absence of BQ123 (1μM). Values are mean±SEM.

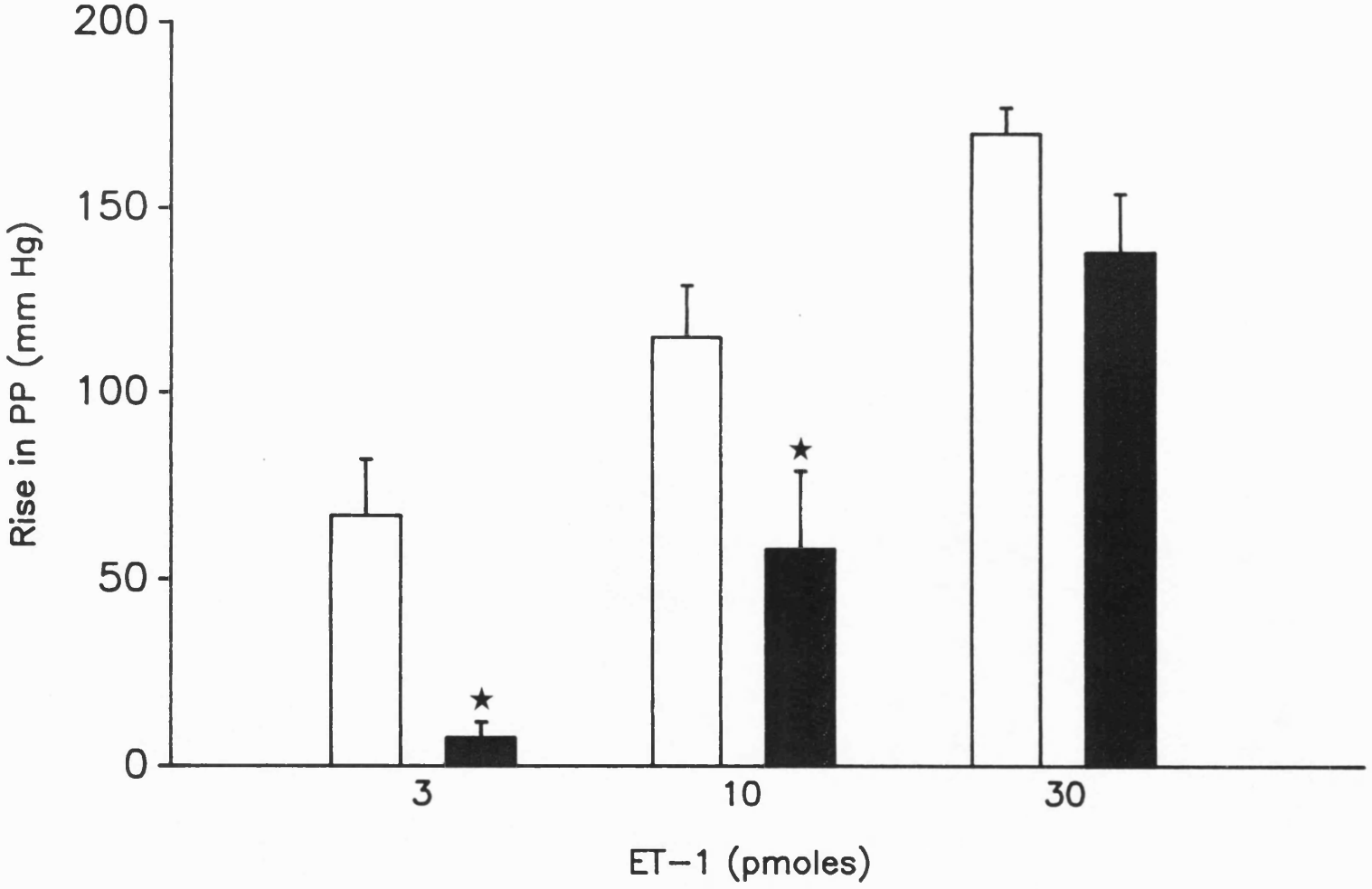

200
150
100
50
0
Rise in PP (mm Hg)
★
★
3
10
30
ET−1 (pmoles)

Figure 15. The time to 75% recovery from peak vasoconstrictor responses to endothelin-1 (ET-1) and ET-3 (both 30pmoles) in the isolated perfused rat kidney in the absence (open bars) (n=6) or presence (closed bars) (n=6) of BQ123 (1μM). ★ $P<0.01$ with respect to ET-1 (30pmoles) in the absence of BQ123 (1μM). Values are mean±SEM.

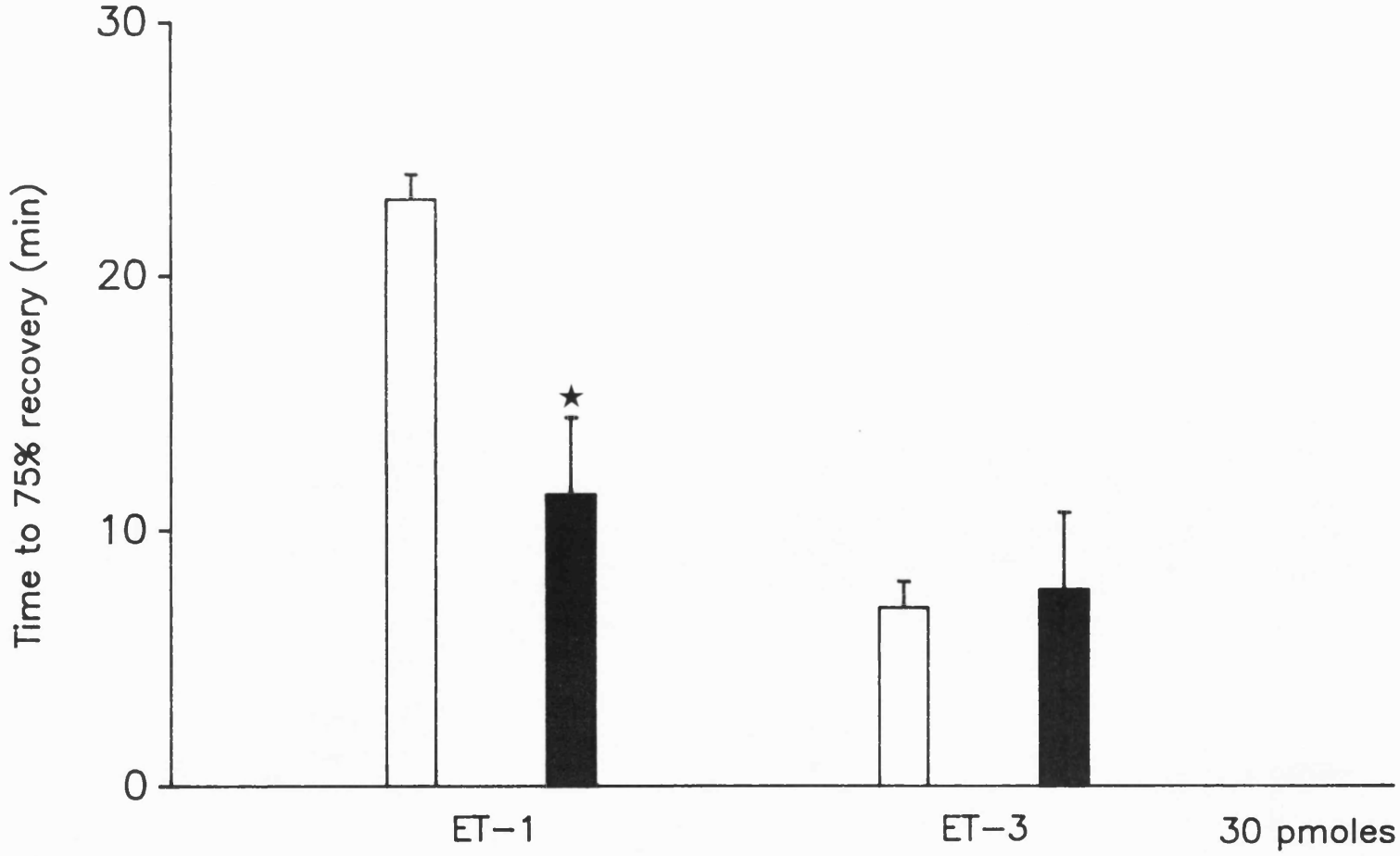
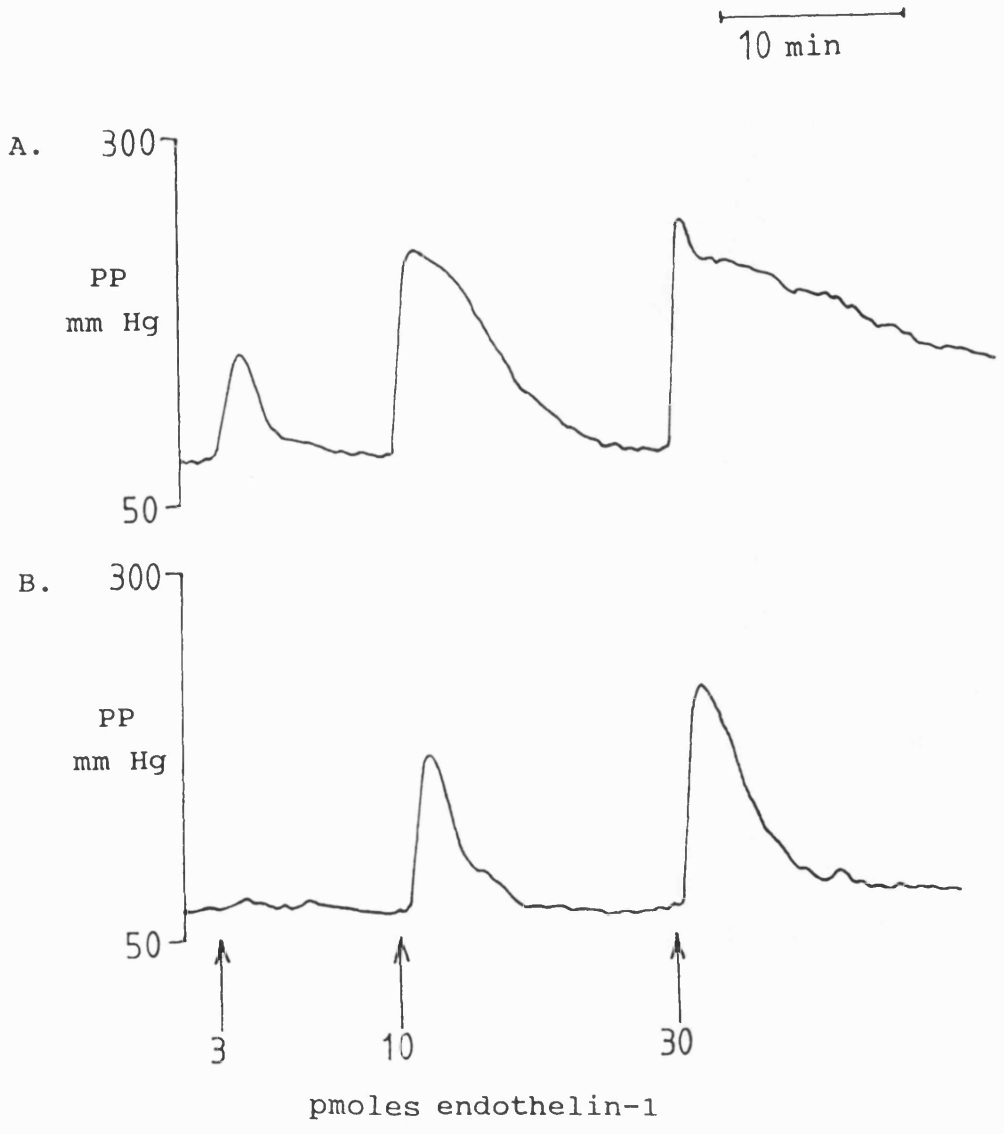

30
20
10
0
Time to 75% recovery (min)
★
ET−1
ET−3
30 pmoles

Figure 16. Experimental trace showing rises in perfusion pressure (PP) due to endothelin-1 (ET-1) (3-30pmoles) in the isolated perfused rat kidney in the absence (A) and presence (B) of BQ123 (1μM).


10 min
A.
300
PP
mm Hg
50
B.
300
PP
mm Hg
50
3
10
30
pmoles endothelin-1

were not significantly different. Peak vasoconstriction to ET-3 (3-30pmoles) was not significantly affected by BQ123(1μM) (Figure 17).

3.7. Effect of indomethacin on renal vasoconstriction to ET-1 and ET-3

Indomethacin(10μM) showed no effect either on basal perfusion pressure or the non-specific vasoconstriction to KCl(110mM). Basal perfusion pressure in the presence and absence of indomethacin(10μM) was 78±4 (n=6) and 76±4 mmHg (n=6), respectively ($P>0.05$). KCl(110mM) raised perfusion pressure by 119±8 (n=6) and 115±10 mmHg (n=6) in the presence and absence of indomethacin(10μM) ($P>0.05$). Neither peak vasoconstriction to ET-1 (Figure 18) nor ET-3 (Figure 19) (3-30pmoles) was significantly affected by indomethacin(10μM). The time to 75% recovery from the peak rise in perfusion pressure due to ET-1(30 pmoles) (23.2±4.5 min, n=6) remained significantly greater than that for ET-3 (30pmoles) (9.7±2.0 min, n=6) in the presence of indomethacin(10μM) ($P<0.05$).

Figure 17. Rises in perfusion pressure (PP) to endothelin-3 (ET-3) in the isolated perfused rat kidney in the absence (open bars) (n=6) or presence (closed bars) (n=6) of BQ123 (1μM). Values are mean±SEM.

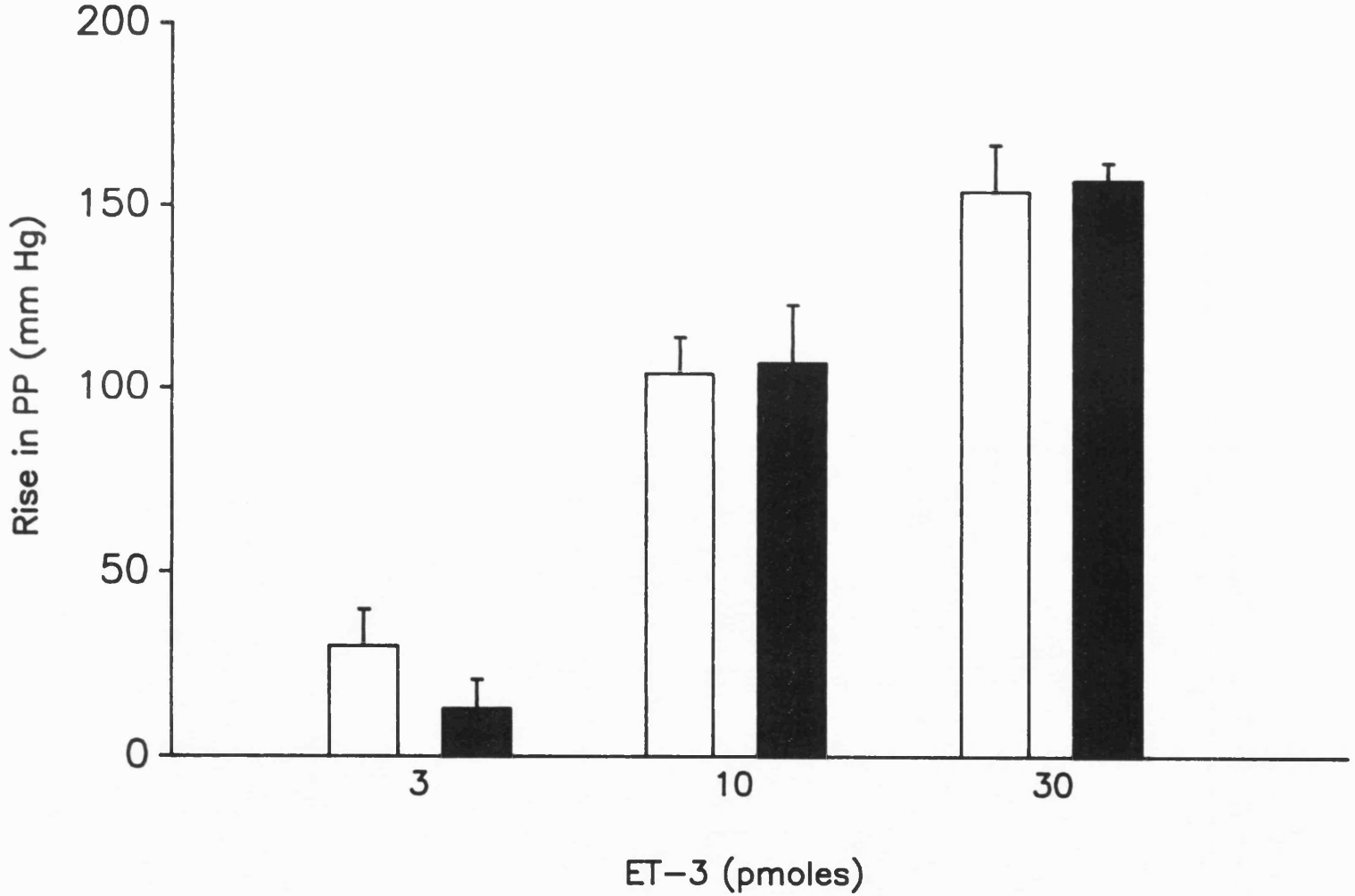

200
150
100
50
0
Rise in PP (mm Hg)
3
10
30
ET–3 (pmoles)

Figure 18. Rises in perfusion pressure (PP) due to endothelin-1 (ET-1) in the isolated perfused rat kidney in the absence (open bars) (n=6) or presence (closed bars) (n=6) of indomethacin (10μM). Values are mean±SEM.

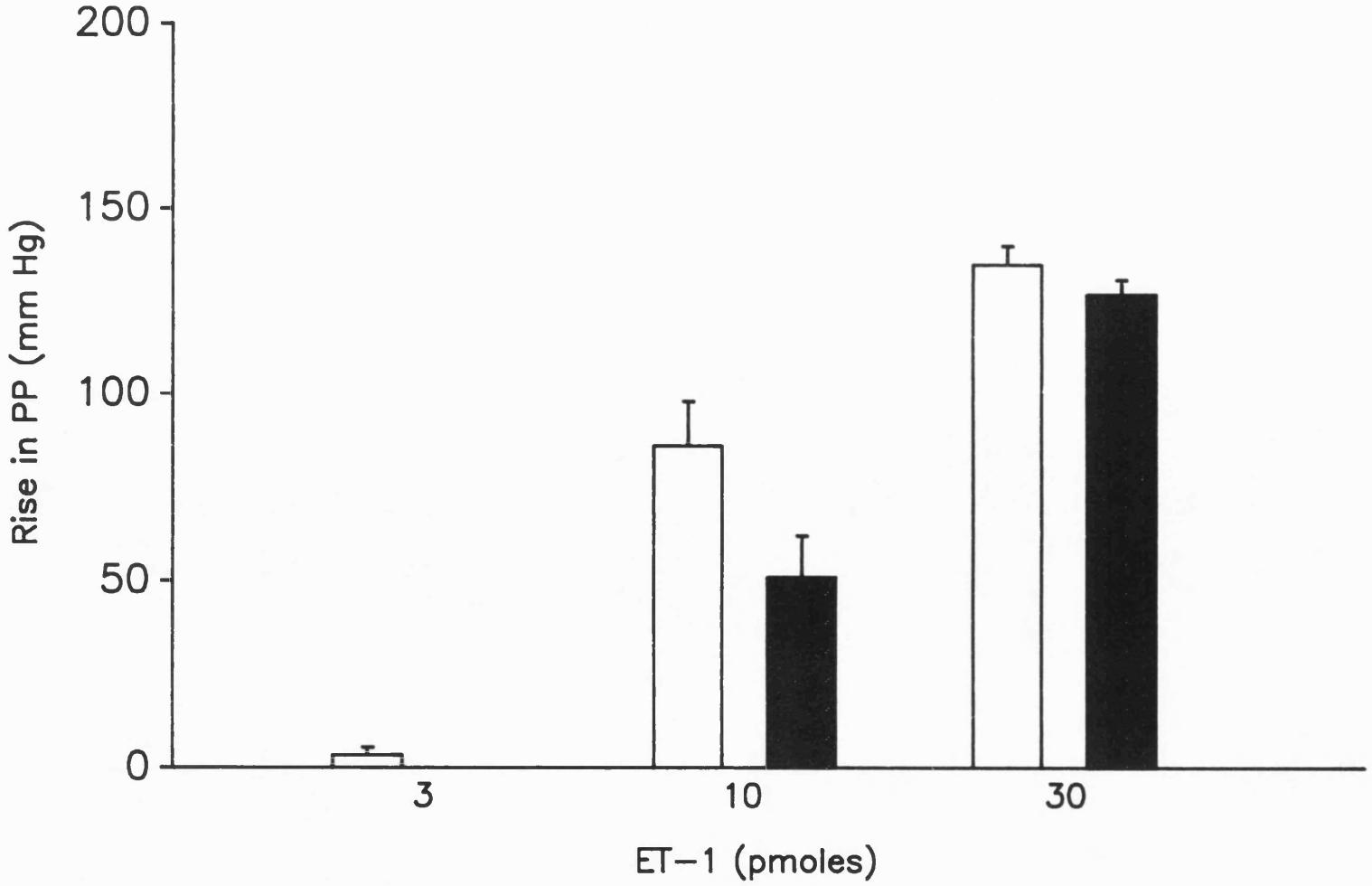
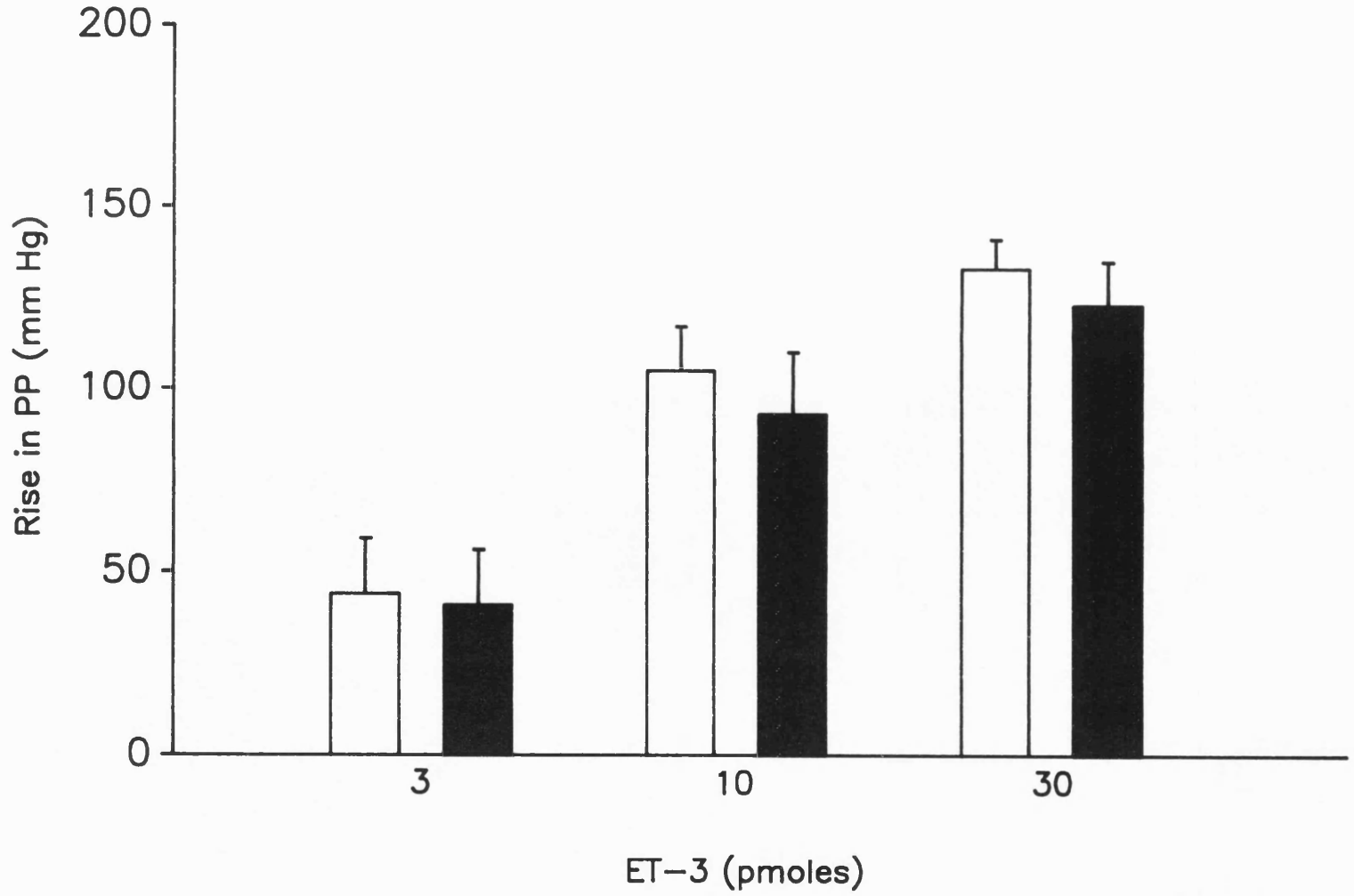

200
150
100
50
0
Rise in PP (mm Hg)
3
10
30
ET−1 (pmoles)

Figure 19. Rises in perfusion pressure (PP) due to endothelin-3 (ET-3) in the isolated perfused rat kidney in the absence (open bars) (n=6) or presence (closed bars) (n=6) of indomethacin (10μM). Values are mean±SEM.


200
150
100
50
0
Rise in PP (mm Hg)
3
10
30
ET–3 (pmoles)

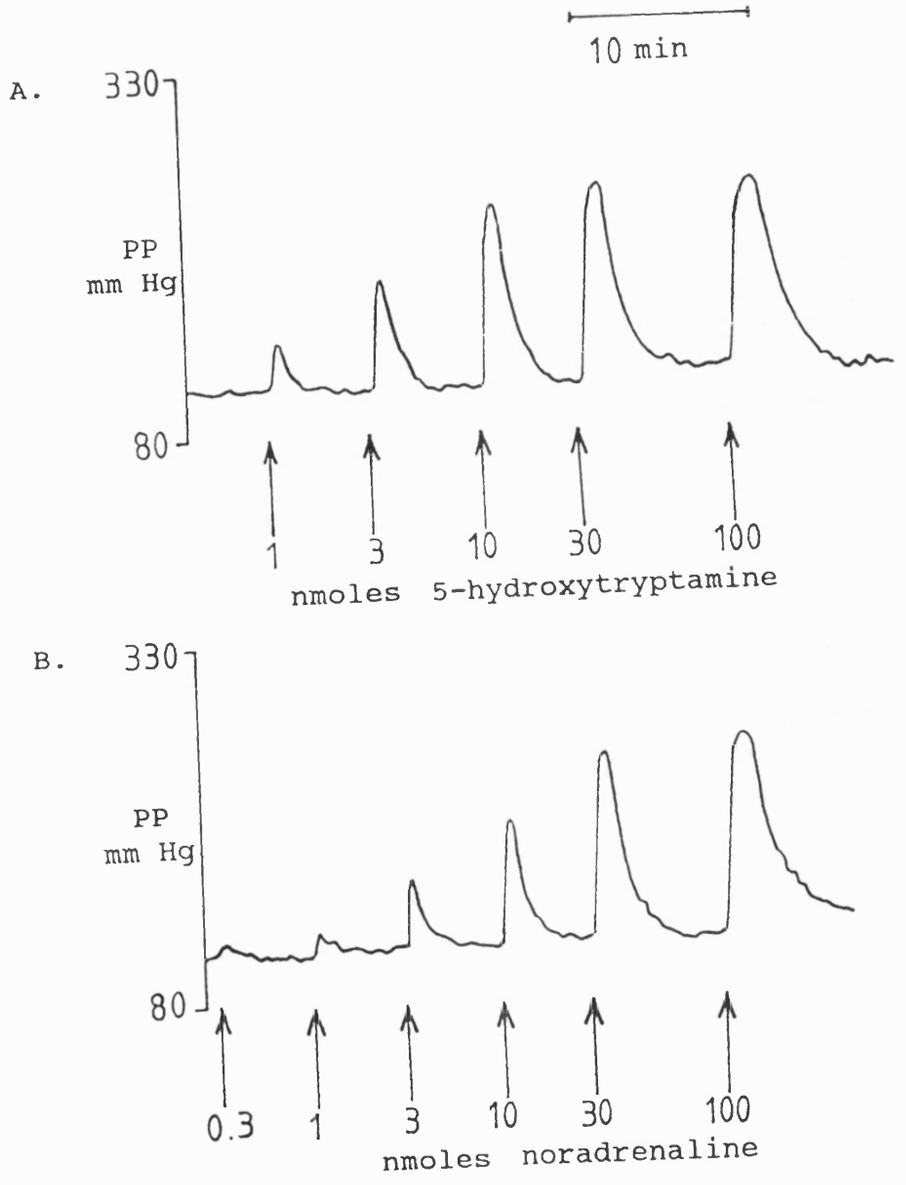
3.8. Effects of renal ischaemia/reperfusion in vivo on renal vasoconstriction to noradrenaline, 5-hydroxytryptamine and KCl

Dose-response series were generated in the isolated perfused rat kidney to noradrenaline (1-300nmoles) and 5-hydroxytryptamine (1-100nmoles) after 30min renal ischaemia with or without 15min reperfusion *in vivo*. Dose-response series to noradrenaline (1-300nmoles) or 5-hydroxytryptamine (1-100nmoles) were repeated 1h later to assess reproducibility. Isolated perfused rat kidneys that were normoperfused *in vivo* for 30min or 45min served as time-matched control preparations (see Figure 20).

3.9. Effect of 30min renal ischaemia in vivo on renal vasoconstriction to noradrenaline, 5-hydroxytryptamine and KCl

Basal perfusion pressure was initially 78±3 (n=8) and 83±4 mmHg (n=6) in the 30min renal ischaemia and control group, respectively ($P>0.05$). At the end of some 2h perfusion, basal perfusion pressure was 61±3 (n=8) and 77±4 mmHg (n=6) in the 30min renal ischaemia and control group, respectively ($P>0.05$). The water content of isolated perfused rat kidneys in each group after this time was 85.5±0.4% which was significantly higher than

Figure 20. Experimental trace showing rises in perfusion pressure (PP) due to (A) 5-hydroxytryptamine (1-100nmoles) and (B) noradrenaline (1-300nmoles) in the isolated perfused rat kidney previously normoperfused *in vivo*.


10 min
A.
330
PP
mm Hg
80
1
3
10
30
100
nmoles 5-hydroxytryptamine
B.
330
PP
mm Hg
80
0.3
1
3
10
30
100
nmoles noradrenaline

the water content of nonperfused kidneys (76.7±0.2%, n=24, P<0.01).

The ED_{50} for noradrenaline (1-300nmoles) during the initial dose-response series *in vitro* after 30min renal ischaemia *in vivo* was 2-fold higher (11.4±2.0 nmoles, n=8) than in the normoperfused control group (5.1±2.0 nmoles, n=6), but statistical significance was not attained (Figure 21). However, when a second dose-response series to noradrenaline (1-300nmoles) was repeated 1h later in the 30min renal ischaemia group, the ED_{50} was found to have significantly decreased with time from 11.4±2.0nmoles to 2.6±2.0nmoles (P<0.025, n=8). In contrast, the ED_{50} for noradrenaline (1-300nmoles) in the control group was not found to significantly vary between the initial and repeated dose-response series. Vasoconstriction to noradrenaline (1-300nmoles) was similar in the 30min ischaemia and control groups during the repeated dose-response series 1h later (Figure 22).

To investigate whether the apparently depressed potency of noradrenaline observed during the initial dose-response series in vitro after 30min renal ischaemia *in vivo* might be selective for this agonist, an initial dose-response series to 5-hydroxytryptamine (1-100nmoles) was generated. 30min renal ischaemia *in vivo* showed no effect to depress vasoconstriction to 5-hydroxytryptamine (1-100nmoles) when this agonist rather than noradrenaline

Figure 21. Rises in perfusion pressure (PP) due to noradrenaline (NA) during the initial dose-response series in the isolated perfused rat kidney after 30min renal ischaemia (open symbols) (n=8) or normoperfusion (closed symbols) (n=6) *in vivo*. Values are mean±SEM.

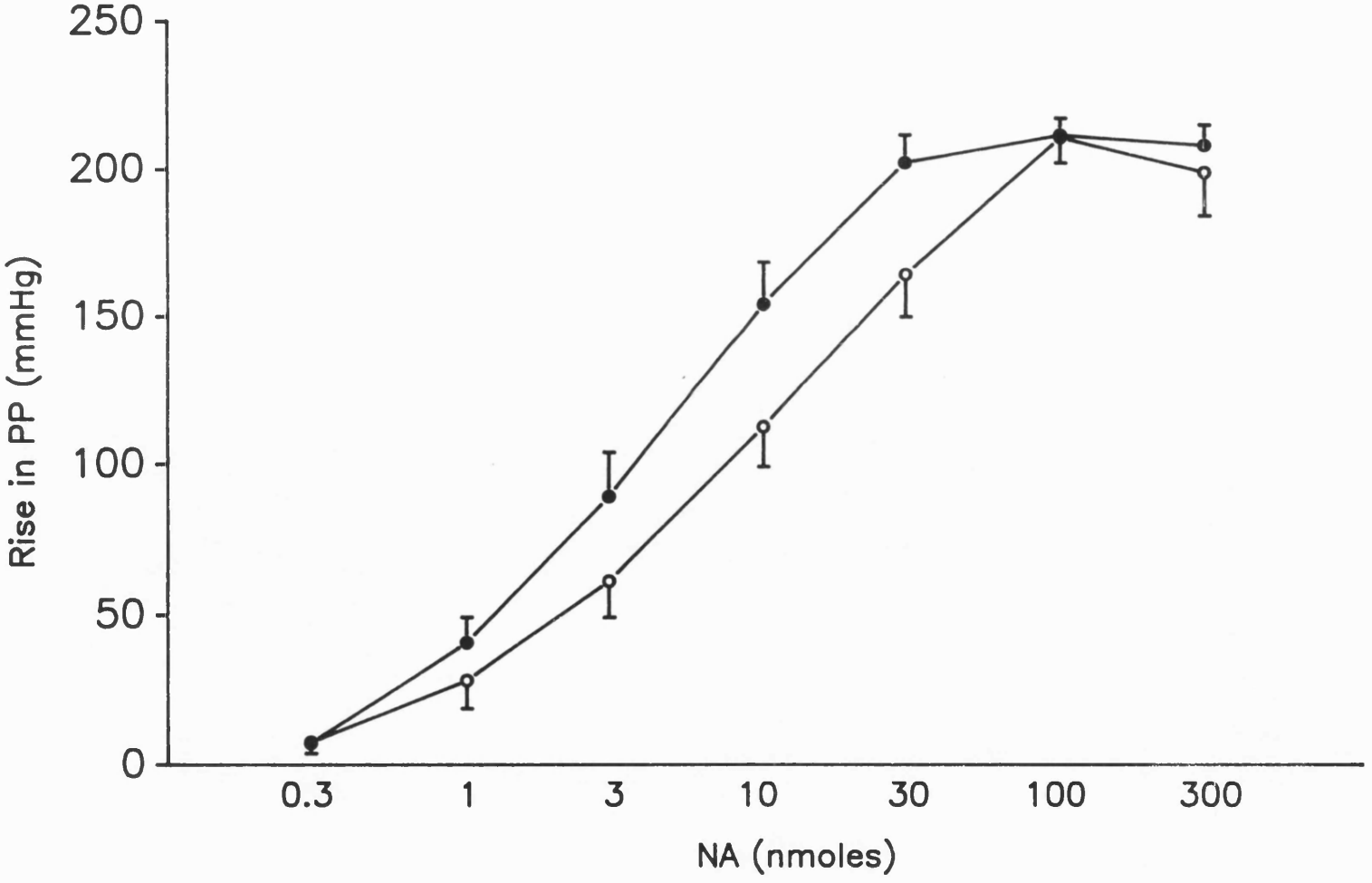
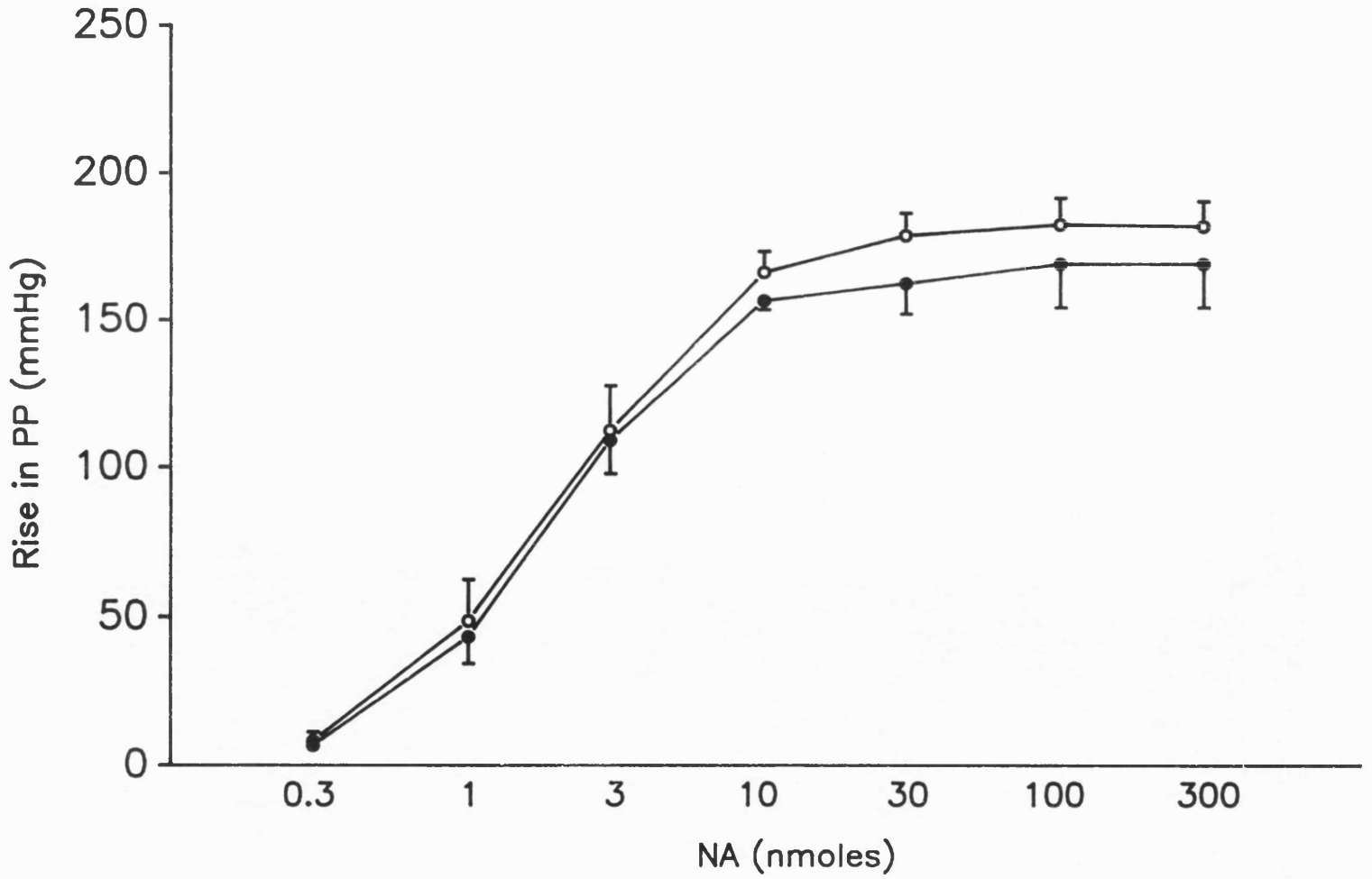

250
200
150
100
50
0
Rise in PP (mmHg)
0.3
1
3
10
30
100
300
NA (nmoles)

Figure 22. Rises in perfusion pressure (PP) due to noradrenaline (NA) during the second dose-response series in the isolated perfused rat kidney after 30min renal ischaemia (open symbols) (n=8) or normoperfusion (closed symbols) (n=6) *in vivo*. Values are mean±SEM.


250
200
150
100
50
0
Rise in PP (mmHg)
0.3
1
3
10
30
100
300
NA (nmoles)

was employed in the initial dose-response series (Figure 23). The ED_{50} for 5-hydroxytryptamine was identical in the 30min renal ischaemia group (4.0±1.0nmoles, n=4) and control group (4.0±0.9nmoles, n=4) (P>0.05) during the initial dose-response series. Moreover, the potency of 5-hydroxytryptamine did not significantly vary *in vitro* with time (1h) in the 30min renal ischaemia group. In addition, 30min renal ischaemia *in vivo* had no significant effect on the non-specific vasoconstriction to KCl (30mM). KCl (30mM) in the 30min renal ischaemia and control group raised perfusion pressure by 74±4 (n=6) and 63±6 mmHg (n=6), respectively (P>0.05).

3.10. Effect of 30min renal ischaemia and 15min reperfusion in vivo on renal vasoconstriction to noradrenaline

Vasoconstriction to noradrenaline (1-300nmoles) examined during the initial dose-response series after 30min renal ischaemia/15min reperfusion was similar to that after 45min normoperfusion *in vivo* (Figure 24). The ED_{50} for noradrenaline in the 30min renal ischaemia/15min reperfusion and control group was 4.5±1.6 (n=6) and 4.2±1.0nmoles (n=6), respectively (P>0.05), during the initial dose-response series and 3.9±1.6 (n=6) and 2.9±1.0nmoles (n=6), respectively (P>0.05), during the repeated dose-response series 1h later. The ED_{50} for

Figure 23. Rises in perfusion pressure (PP) due to 5-hydroxytryptamine (5-HT) during the initial dose-response series in the isolated perfused rat kidney after 30min renal ischaemia (open symbols) (n=4) or normoperfusion (closed symbols) (n=4) *in vivo*.

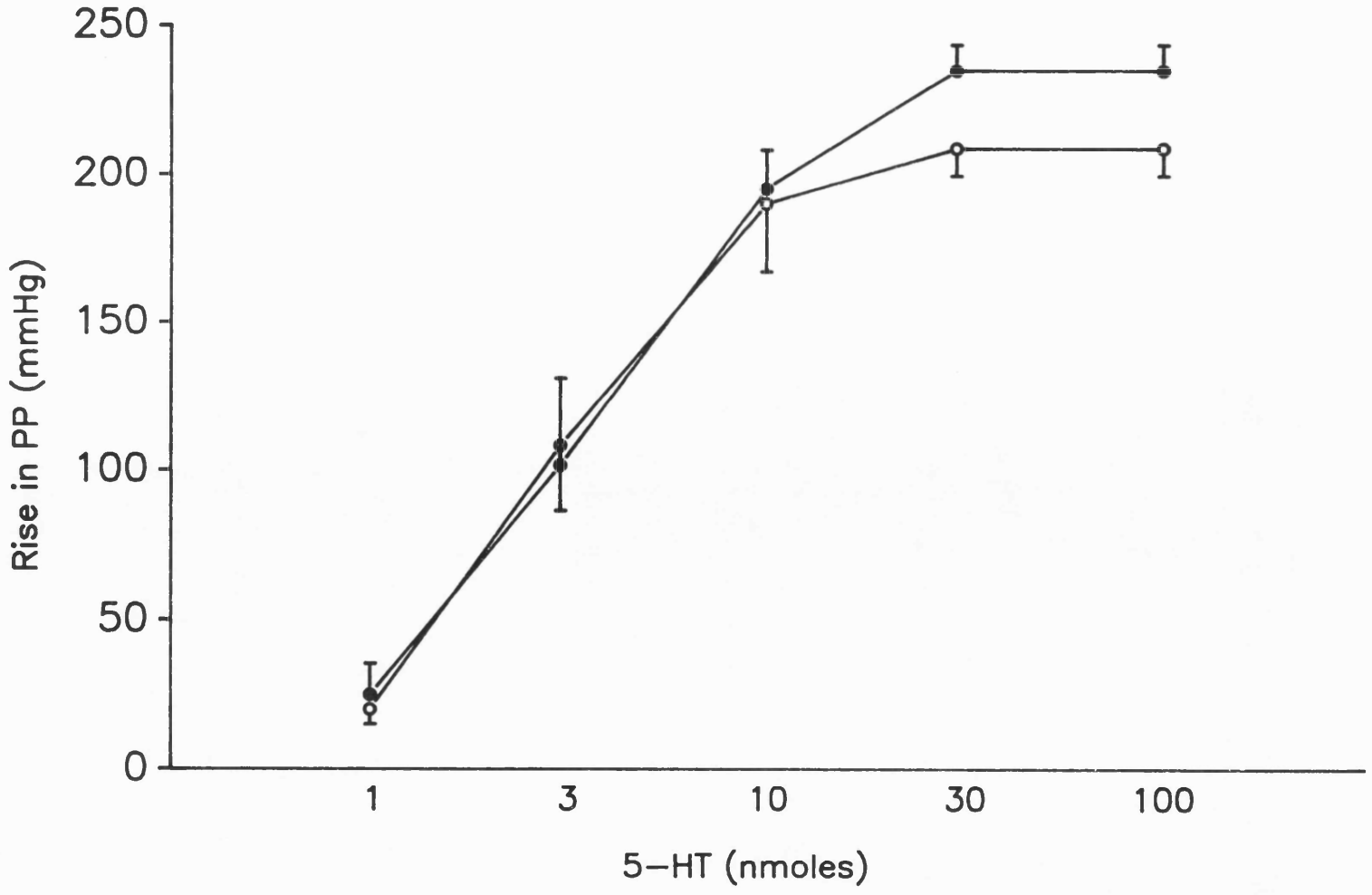
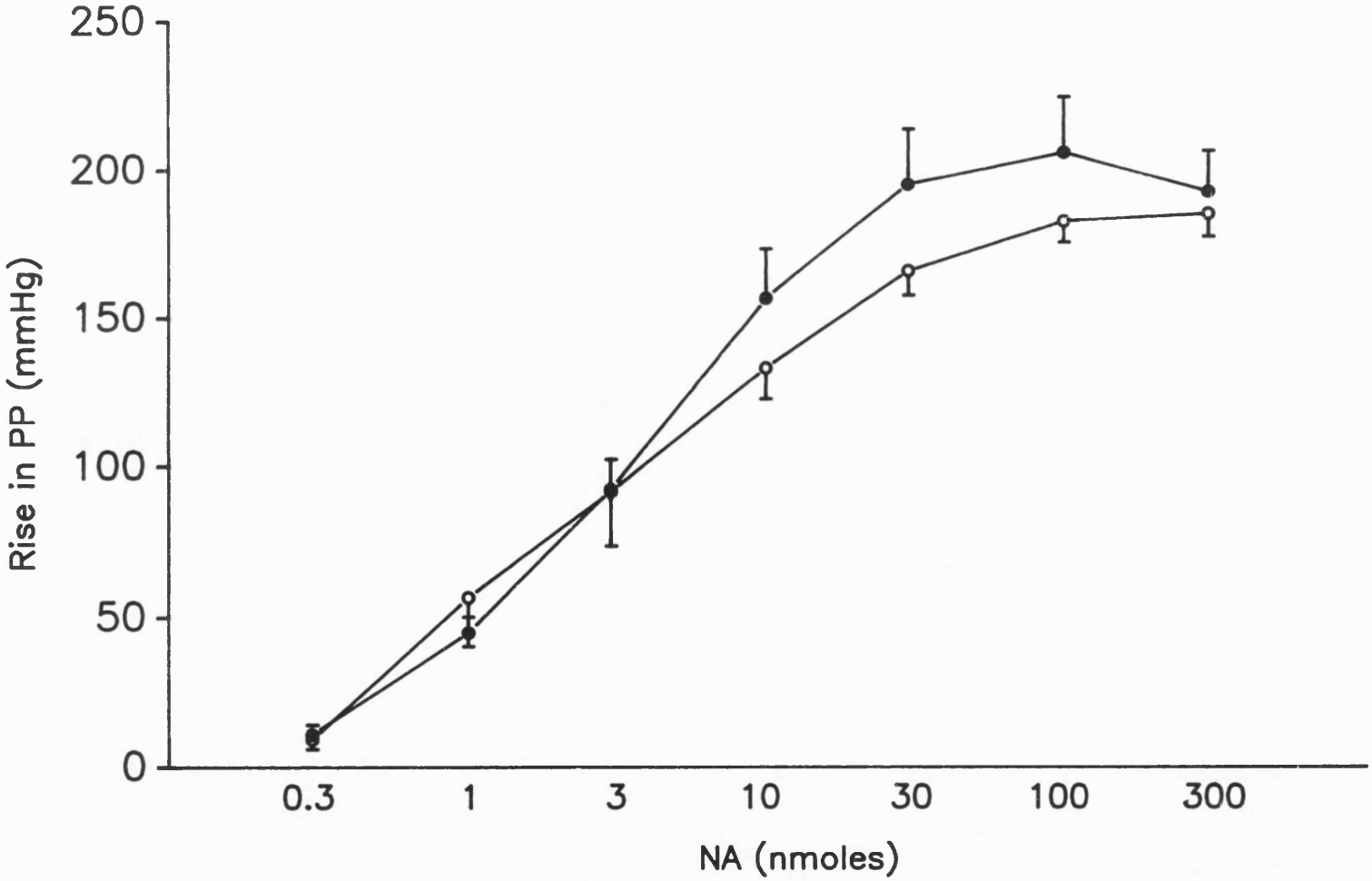

250
200
150
100
50
0
Rise in PP (mmHg)
1
3
10
30
100
5–HT (nmoles)

Figure 24. Rises in perfusion pressure (PP) due to noradrenaline (NA) in the isolated perfused rat kidney during the initial dose-response series after 30min renal ischaemia followed by 15min reperfusion (open symbols) (n=6) or 45min normoperfusion (closed symbols) (n=6) *in vivo*. Values are mean±SEM.


250
200
150
100
50
0
Rise in PP (mmHg)
0.3
1
3
10
30
100
300
NA (nmoles)

noradrenaline in the 30min renal ischaemia/15 min reperfusion group did not significantly vary over 1h perfusion.

3.11. Effect of 30min renal ischaemia in vivo on renal vasoconstriction to ET-1 and ET-3

Basal perfusion pressure in the 30min renal ischaemia and control groups was in the range 70-90 mmHg. 30min ischaemia *in vivo* had no effect on dose-dependent vasoconstrictor responses to ET-1 (3-30pmoles) (Figure 25) or ET-3 (3-30pmoles) (Figure 26). Furthermore, the time to 75% recovery from peak vasoconstrictor responses to ETs was unaffected by previous 30min renal ischaemia *in vivo*.

3.12. Assessment of MPO activity

In order to investigate the presence of accumulated PMNs after renal ischaemia/reperfusion *in vivo*, renal MPO activity was assessed as a biochemical marker of PMNs.

Figure 25. Rises in perfusion pressure (PP) due to endothelin-1 (ET-1) in the isolated perfused rat kidney after 30min renal ischaemia (closed bars) (n=6) or normoperfusion (open bars) (n=6) *in vivo*. Values are mean±SEM.

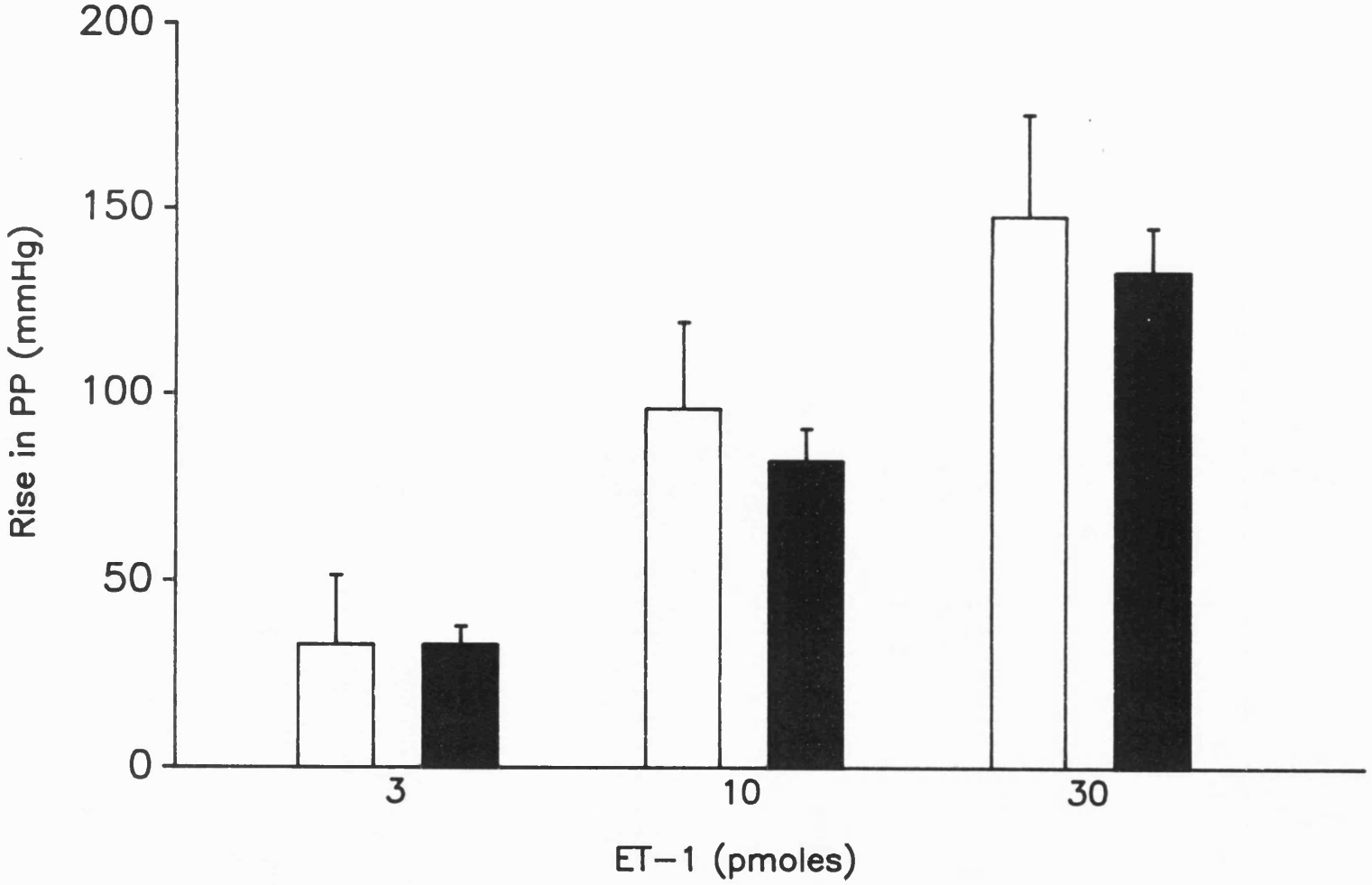
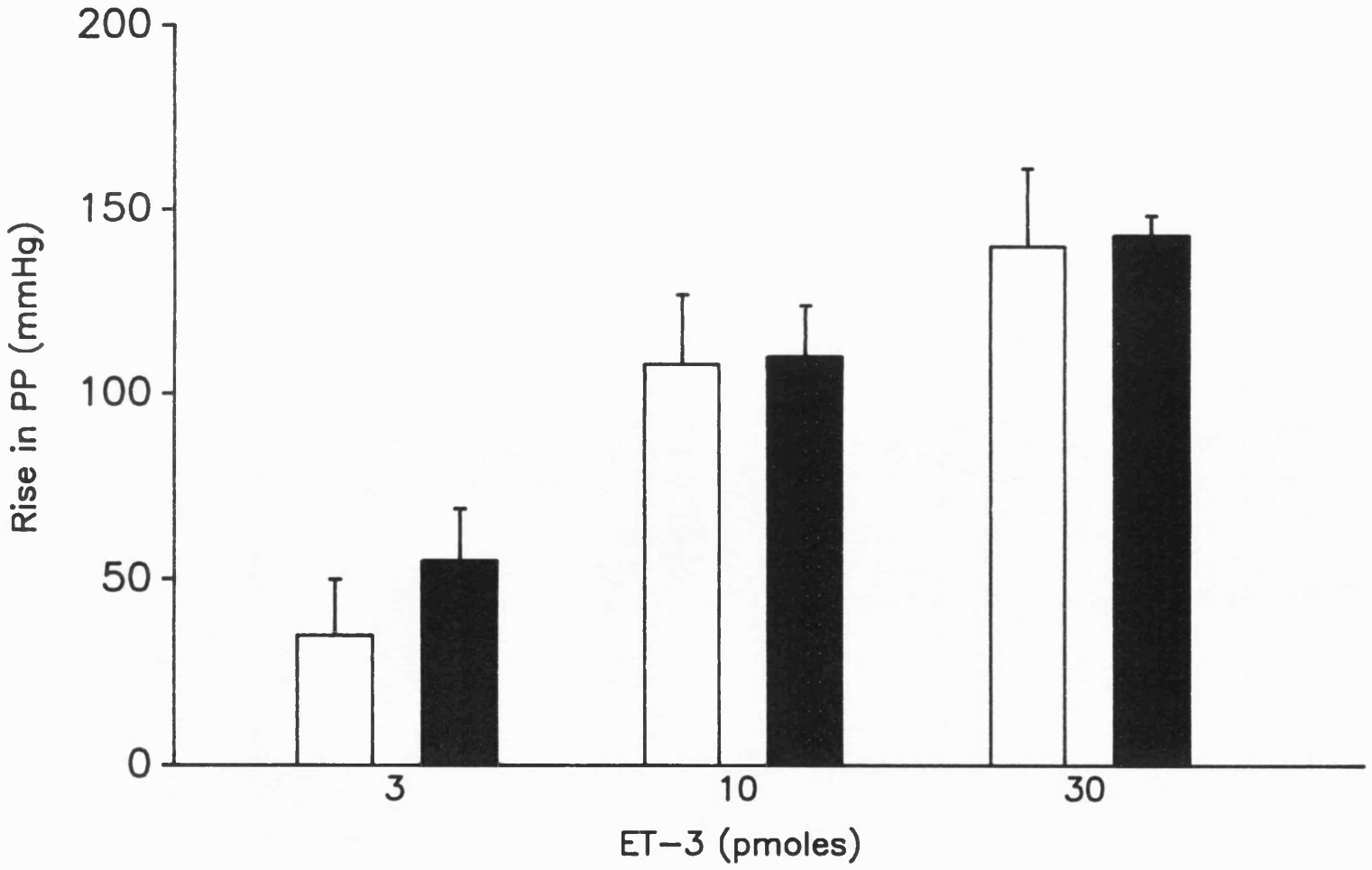

200
150
100
50
0
Rise in PP (mmHg)
3
10
30
ET–1 (pmoles)

Figure 26. Rises in perfusion pressure (PP) due to endothelin-3 (ET-3) in the isolated perfused rat kidney after 30min renal ischaemia (closed bars) (n=6) or normoperfusion (open bars) (n=6) *in vivo*. Values are mean±SEM.


200
150
100
50
0
Rise in PP (mmHg)
3
10
30
ET−3 (pmoles)

3.13. Assay of MPO derived from rat blood PMNs

MPO activity from rat blood PMNs was linearly correlated with PMN number from which MPO was extracted in either an assay using TMB (r=1.000, P<0.01, n=5) or o-dianisidine (r=0.998, P<0.01, n=5) (Figure 27). However, the TMB assay of PMN-derived MPO was 3-fold more sensitive than the o-dianisidine assay (51.83±1.56 δA/min/10^6 PMNs compared to 18.08±1.07 σA/min/10^6 PMNs, P<0.01, n=5). The more sensitive TMB assay was therefore adopted in subsequent studies.

3.14. Recovery of exogenous horseradish activity from rat renal supernate

The initial rate of increase of absorbance at 630nm was linearly correlated with the concentration of horseradish peroxidase in the well (8.7-43.3μg/l). The activity of exogenous horseradish peroxidase added to renal supernate derived from non-ischaemic rat kidneys was significantly depressed (P<0.01, n=4) compared to the activity of exogenous horseradish peroxidase activity added to an equal volume of 0.5% HTAB (Figure 28) and represented a recovery of approximately only 9% (i.e. 91% inhibiiton). The activity of exogenous horseradish peroxidase added to renal supernate incubated at 60°C for 2h was still significantly depressed (P<0.01, n=4). Similarly, when

Figure 27. Myeloperoxidase (MPO) activity derived from rat blood polymorphonucleocytes assessed as the initial rate of change of absorbance (δA/min), using 3,3',5,5'-tetramethylbenzidine (solid line) (n=5) or o-dianisidine (dashed line) assays (n=5). ★P<0.05 with respect to o-dianisidine assay. Values are mean±SEM.

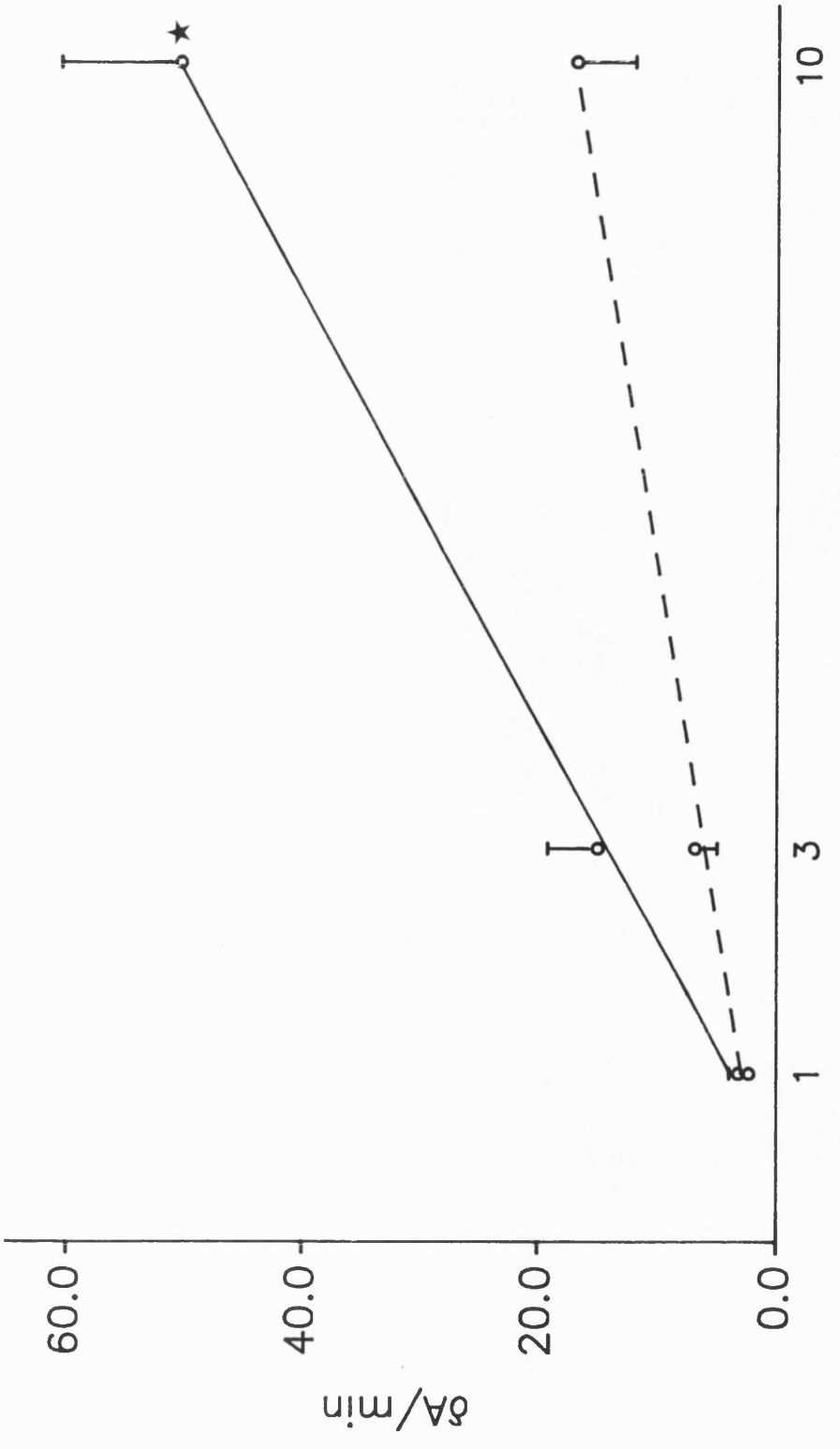
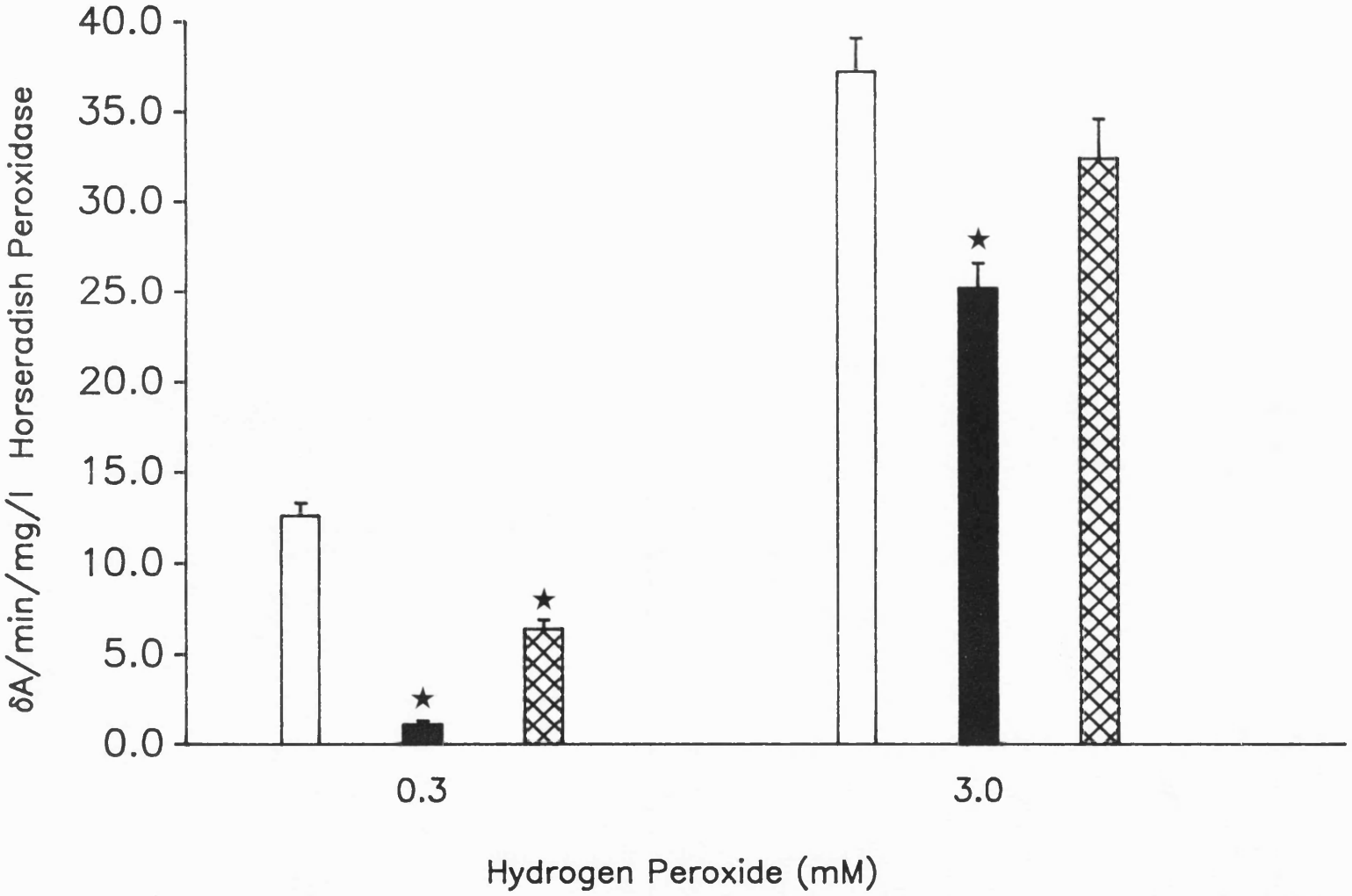

60.0
40.0
20.0
0.0
δA/min
1
3
10
PMN (x10^5)

Figure 28. Horseradish peroxidase activity assessed as the initial rate of change of absorbance (δA/min) per unit concentration of enzyme in the presence of 0.5% (w/v) HTAB/50mM pH6.0 phosphate buffer (control, open bars) (n=4) or rat renal supernate either incubated at 60°C for 2h (cross-hatched bars) (n=4) or unincubated (closed bars) (n=4): effects of 0.3 or 3.0mM hydrogen peroxide in assay. ★$P<0.01$ with respect to appropriate control value. Values are mean±SEM.


40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
δA/min/mg/l Horseradish Peroxidase
0.3
3.0
Hydrogen Peroxide (mM)

H_2O_2 was employed at a 10-fold higher concentration in the assay (3.0mM), the activity of exogenous horseradish peroxidase activity added to untreated renal supernate remained significantly reduced ($P<0.01$, $n=4$). However, when H_2O_2 was present in the assay at 3.0mM and the renal supernate had been heat inactivated, the activity of exogenous horseradish peroxidase activity was not significantly different from the appropriate control activity. No peroxidase activity could be detected in heat-inactivated renal supernate *per se* in the absence of exogenous horseradish peroxidase.

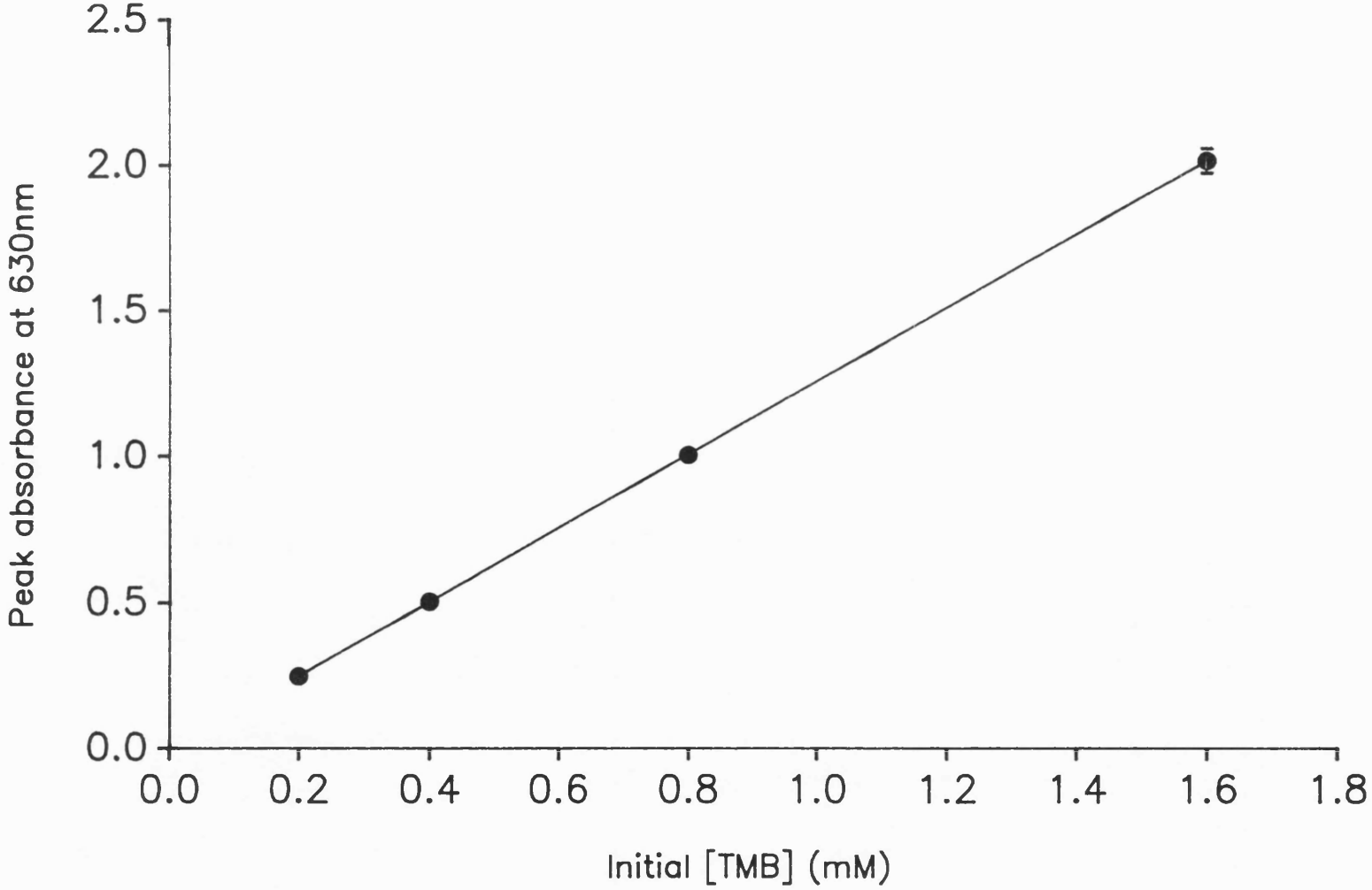
3.15. Determination of molar absorption coefficient for TMB-dehydrogenation product

The peak absorbance values at 630nm were linearly correlated with the initial concentration of TMB (0.2-1.6mM) ($r=1.000$, $P<0.01$, $n=3$) (Figure 29). The molar absorption coefficient for TMB-dehydrogenation product was estimated to be 3152.5±5.0 (n=3).

3.16. Renal MPO activity and wet weight after ischaemia/reperfusion <u>in vivo</u>

When renal supernate was assayed under conditions where the recovery of exogenous horseradish peroxidase activity

Figure 29. Determination of molar absorption coefficient for 3,3',5,5'-tetramethylbenzidine (TMB) dehydrogenation product: peak absorbance (A) at 630nm versus initial concentration of TMB. The reaction was catalysed by excess horseradish peroxidase.


Peak absorbance at 630nm
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Initial [TMB] (mM)

was near 100%, i.e. 2h incubation at 60°C and 3.0 mM assay H_2O_2, kidneys undergoing 45min (but not 30min) ischaemia and 1, 3 or 6h reperfusion *in vivo* showed significant MPO activity (Figure 30), indicating tissue PMN accumulation. MPO activity in sham-operated kidneys was undetectable. Furthermore, MPO activity was undetectable in kidneys undergoing 45min ischaemia and 1 or 3h reperfusion when assayed under conditions where the recovery of exogenous horseradish peroxidase activity was only 9%, i.e. no heat inactivation and 0.3 mM assay H_2O_2 incubation. The wet weight of kidneys undergoing 45min ischaemia and 0.5-6h reperfusion *in vivo* was significantly increased as a percentage of the wet weight of sham-operated kidneys (Figure 31). Left kidney wet weight was 99.8±2.5% of the right kidney wet weight in a random sample of animals (n=6).

3.17. Effects of antibodies against adhesion molecules used by PMNs on renal MPO activity after ischaemia/reperfusion in vivo

Treatment of animals with anti-ICAM-1 or anti-CD18 MoAb (20μg/kg via tail vein both 5min before renal ischaemia and at the commencement of reperfusion was associated with significantly reduced MPO activity in kidneys undergoing 45min ischaemia and 1h reperfusion *in vivo* (Figure 32). In addition, identical treatment with the

Figure 30. Myeloperoxidase (MPO) activity in rat kidneys after 45min renal ischaemia and various periods of reperfusion *in vivo* expressed per unit wet weight of the sham-operated kidney. ★ $P<0.05$ with respect to zero MPO activity in sham-operated kidneys. Values are mean±SEM.

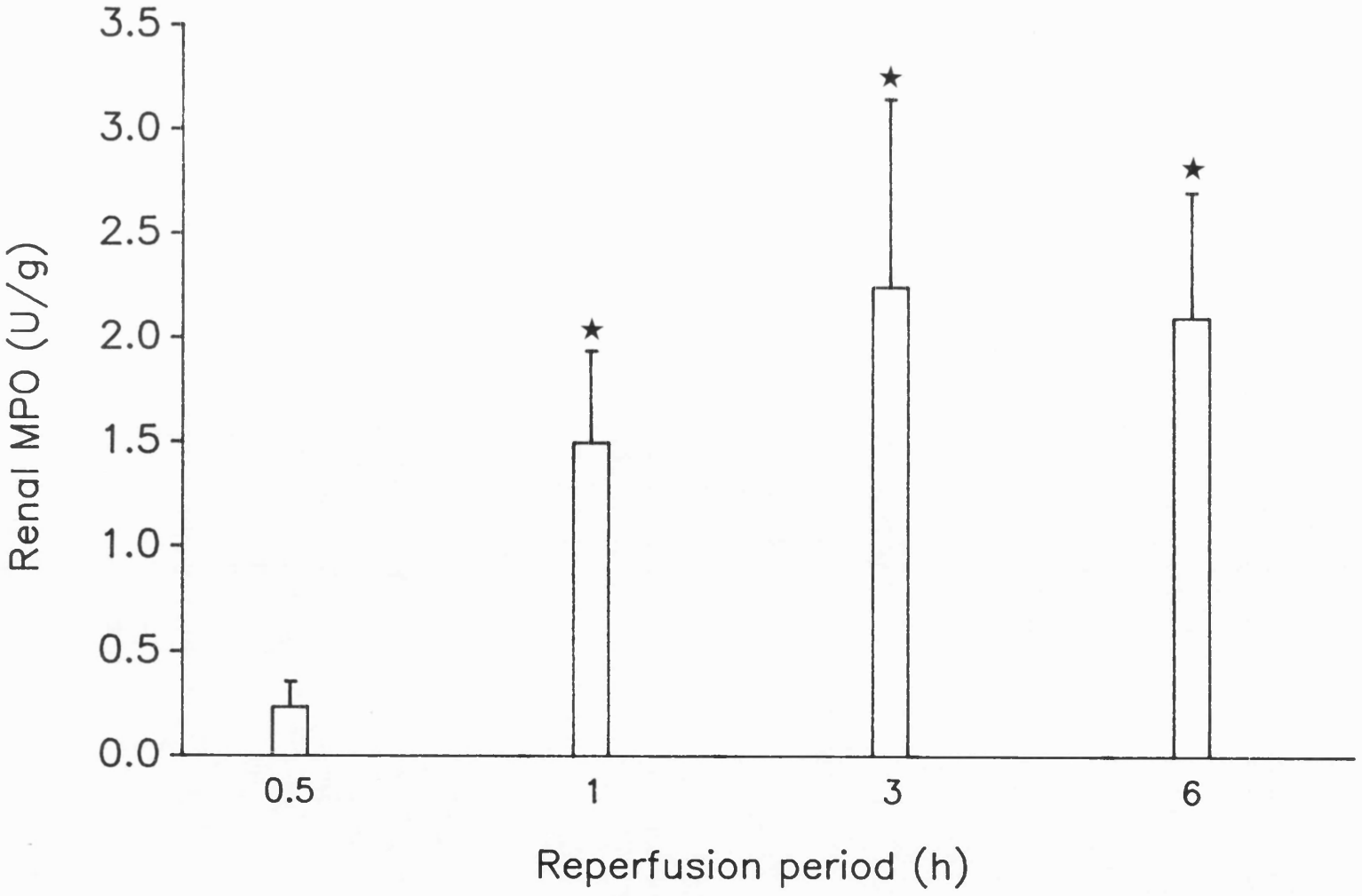

Renal MPO (U/g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.5
1
3
6
Reperfusion period (h)

Figure 31. Wet weight of rat kidneys after 45min renal ischaemia and various periods of reperfusion *in vivo* as a percentage of the wet weight of sham-operated kidneys.

★ $P<0.05$ with respect to 100%. Values are mean±SEM.

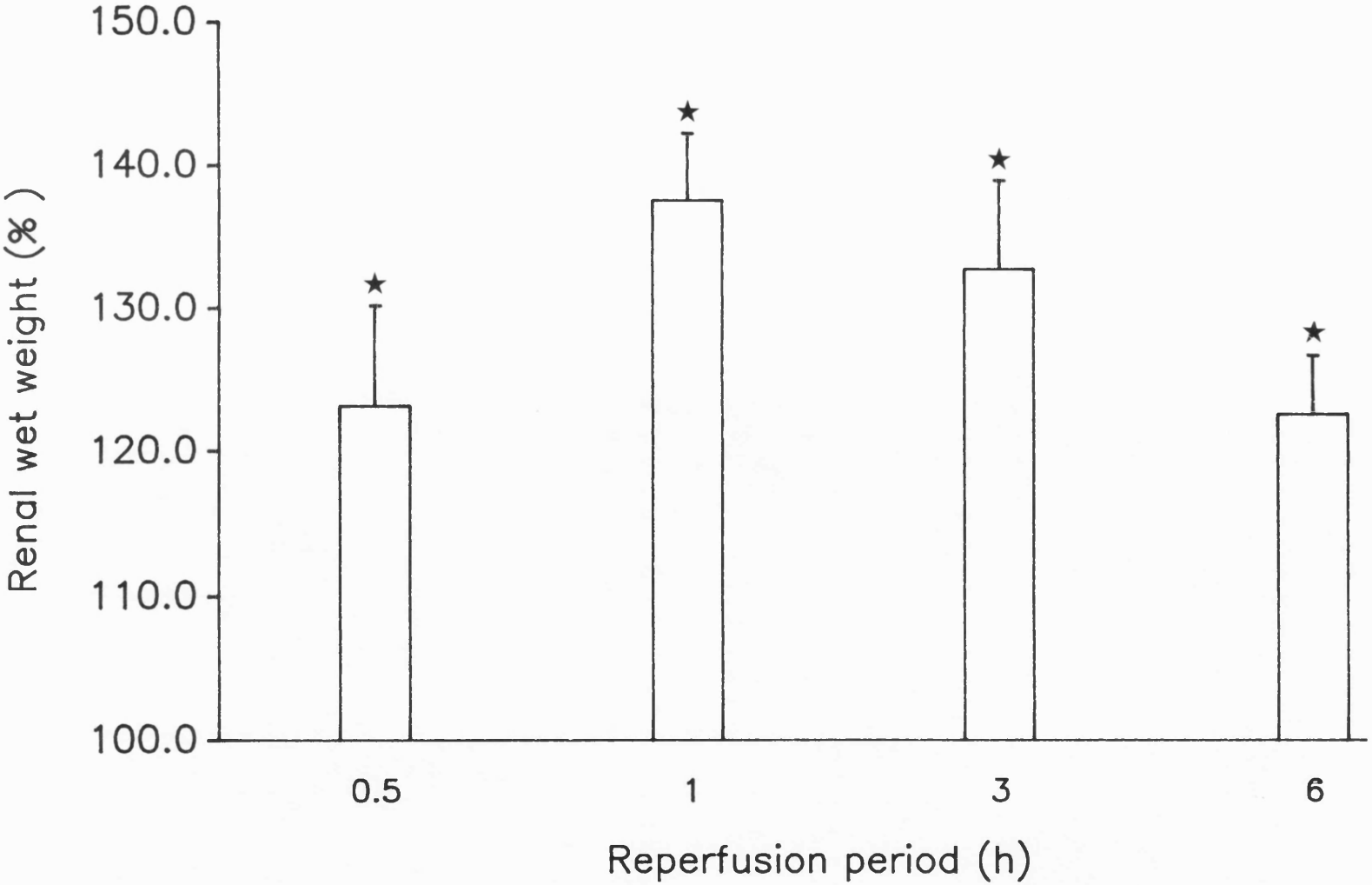
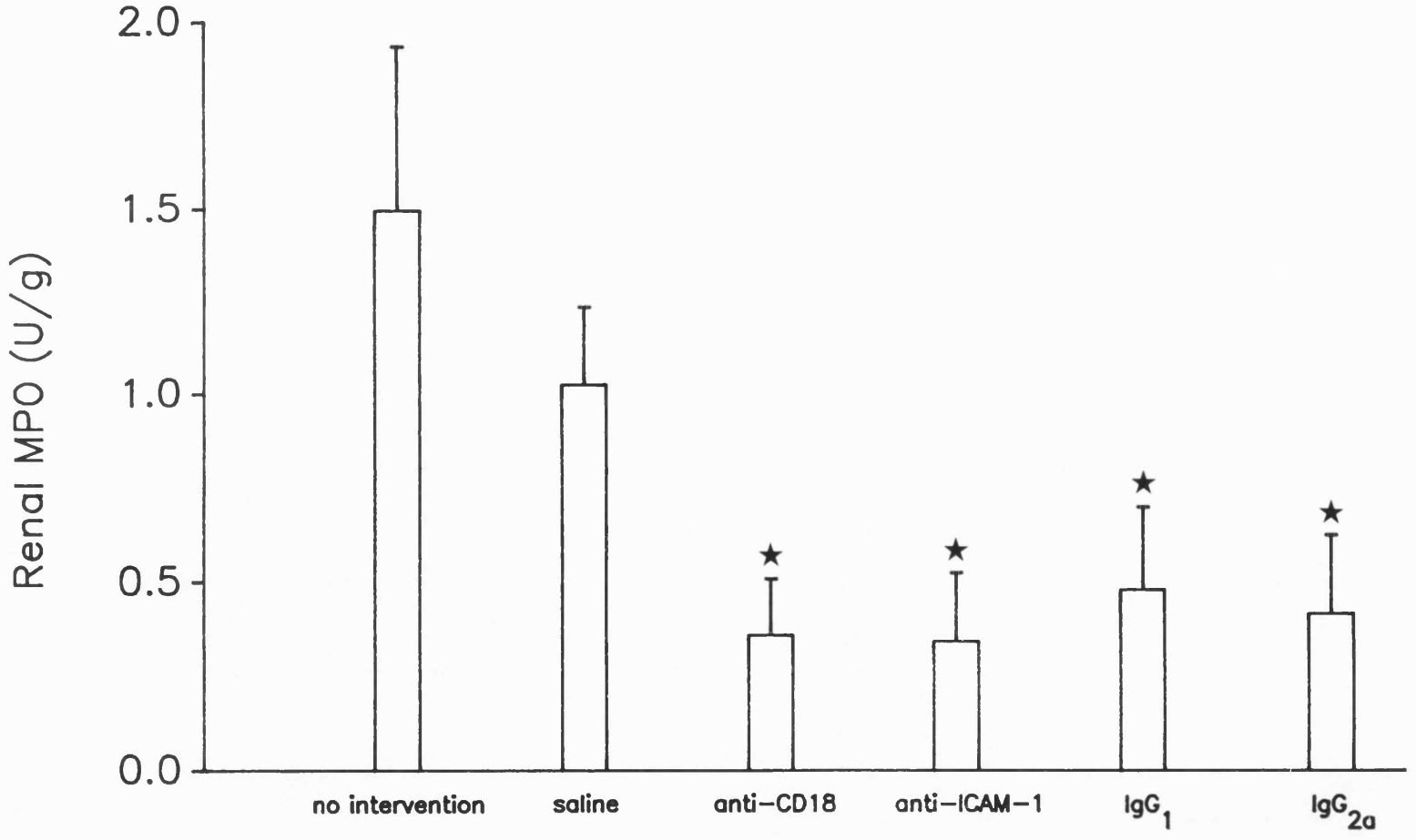

150.0
140.0
130.0
120.0
110.0
100.0
Renal wet weight (%)
0.5
1
3
6
Reperfusion period (h)

Figure 32. Effects of monoclonal antibodies (MoAbs) against rat CD18 of β_2-integrins and rat intercellular adhesion molecule-1 (ICAM-1) or the isotype-matched control antibody murine IgG_1 and an unrelated murine IgG_{2a} antibody or saline vehicle, on myeloperoxidase (MPO) activity in rat kidneys after 45min ischaemia and 1h reperfusion *in vivo*. Antibodies were administered both 5min before renal ischaemia (20μg/kg i.v.) and at the commencement of reperfusion (20μg/kg i.v.). Saline vehicle was administered (0.5ml/kg i.v.) at identical time points. ★$P<0.05$ with respect to ischaemic/reperfused renal MPO activity with no intervention (control). Values are mean±SEM.


Renal MPO (U/g)
2.0
1.5
1.0
0.5
0.0
no intervention
saline
anti-CD18
anti-ICAM-1
IgG1
IgG2a

isotype-matched control antibody murine IgG_1 or an unrelated murine IgG_{2a} antibody produced a similar effect. In contrast, MPO activity in kidneys undergoing 45min ischaemia and 1h reperfusion in animals receiving vehicle alone *in vivo* (0.5ml/kg via tail vein of 0.9% (w/v) saline) was not significantly affected. MPO activity in sham-operated kidneys was undetectable in these experiments. Antibody interventions (40μg/kg i.v. total) did not significantly affect increases in the wet weight of rat kidneys undergoing 45min ischaemia and 1h reperfusion *in vivo*.

3.18. Effect of murine IgG_1 on recovery of exogenous horseradish peroxidase activity from rat renal supernate

To investigate whether a murine antibody preparation could directly affect the photometric assay of peroxidase, the recovery of exogenous horseradish peroxidase activity added to rat renal supernate derived from non-ischaemic kidneys exposed to murine IgG_1 (40μg/kg i.v. total) *in vivo* was assessed. The recovered activity was not significantly different from 100%.

3.19. FACS analysis

In an investigation of whether murine antibodies could non-specifically recognise a common binding site on the surface of PMNs, a FACS analysis demonstrated a clear dextral shift in the the population density binding anti-CD18 MoAb relative to anti-ICAM-1 MoAb or murine IgG_1 or IgG_{2a} antibodies (Figure 33).

Figure 33. Fluorescence activated cell sorting (FACS) analysis of binding of murine antibodies to rat blood polymorphonucleocytes.

LEGEND

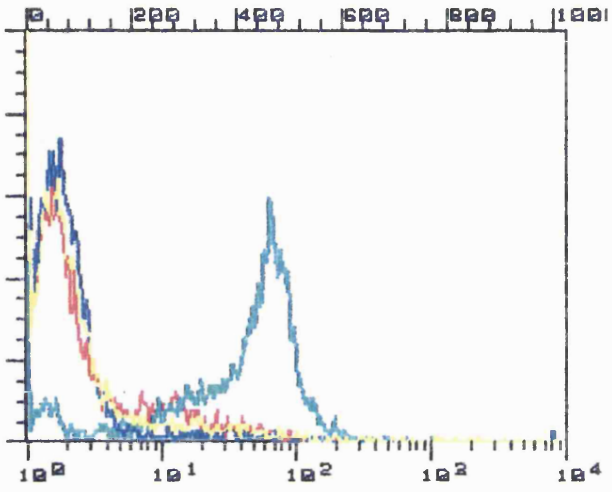
green: anti-CD18

yellow: anti-ICAM-1

blue: IgG_1

red: IgG_{2a}

Fluorescence



Cell density

4. Discussion

4.1. Vasodilation to histamine in the isolated perfused rat kidney: roles of NO, cyclo-oxygenase products and H_1, H_2 and H_3 receptors

The reason(s) for the often unstable nature of methoxamine-induced renal vascular tone observed during studies of vasodilator reactivity, may possibly include: an α_1-adrenoceptor-mediated variable release of vasoactive prostaglandins (Cooper & Malik, 1985); a heterogenous and variable dilator response of the renal vasculature to α_1-adrenoceptor-mediated rises in renal perfusion pressure (Burton et al., 1989); and a renal autoregulatory capacity (see Vander, 1971; de Wardener, 1985). Those periods during this study when methoxamine-induced renal vascular tone was reproducibly stable, may conceivably be accounted for by a seasonal variability in the animals and/or a change in animal site services.

Histamine acted as a dilator of the rat renal microvasculature when vascular tone was induced by preconstriction. The reason for the enhanced efficacy of histamine and acetylcholine as vasodilators when tone was induced with methoxamine rather than potassium is unclear. However, differences in non-specific vasodilator reactivity can probably be ruled out since reactivity to

the directly acting vasodilator papaverine was equivalent *vis-à-vis* potassium- and methoxamine-induced tone. One possibility for both histamine and acetylcholine is that these vasodilator agonists are able to differentiate between agonist- and depolarisation-induced vascular tone at the second messenger level (see van Breeman et al., 1986; Nishimura et al., 1989). Potassium depolarisation has previously been shown to inhibit responses to acetylcholine (Noll et al., 1991) while the second messenger associated with EDRF(NO), cyclic guanosine monophosphate (cGMP) (Murad et al., 1985; Rapoport & Murad, 1983) has been reported to be less effective in reversing vascular tone due to depolarisation than to α-adrenoceptor stimulation (Lincoln et al., 1983; Hester et al., 1979; 1988). Another consideration concerns a potentially heterogenous effect of both contractile agonists and vasodilators in terms of which renal resistance vessels are preferentially preconstricted by the former and which resistance vessels are preferentially dilated by the latter (Cairns et al., 1989; 1991). One additional possibility for histamine, is that methoxamine-induced tone may permit indirect vasodilation due to a histamine-stimulated release of catecholamines from adrenergic nerve terminals (Balfagon et al., 1984); while depolarisation with potassium might be expected to stimulate the release of adrenergic catecholamines *per se* and thereby deny this mechanism to histamine.

The pA_2 value of 6.67 estimated for ranitidine in the present study from a functional inhibition curve, is in close agreement with the pA_2 (6.70) obtained for this H_2 receptor antagonist by Cavanagh et al. (1983) in the guinea-pig atrium using Schild analysis. Furthermore, vasodilation to histamine (30nmoles) was effectively abolished by ranitidine (10μM) while vasodilation to an equieffective dose of papaverine (30nmoles) was unaffected. These findings clearly implicate predominantly H_2 receptors in the subservance of vasodilation to histamine in the isolated perfused rat kidney. The finding that vasodilation to histamine was also partially sensitive to mepyramine (1μM) suggests that H_1 stimulation also promotes renal vasodilation while the ineffectiveness of thioperamide (10μM) provides evidence that H_3 receptors are not involved. The use of selective agonists at: H_1 receptors, such as 2-pyridylethylamine and 2-methylhistamine; H_2 receptors, such as dimaprit and impromodine; and at H_3 receptors, such as R(-)-α-methylhistamine (see Hill, 1987; Arrang et al., 1987), would help to further elucidate the roles of these receptors in renal vasodilation to histamine.

L-NAME (0.3mM) and L-NOARG (0.3mM), failed to reduce vasodilation to histamine in the isolated perfused rat kidney, preconstricted either by depolarisation or by α_1-adrenoceptor stimulation. Both L-NOARG and L-NAME are potent inhibitors of NO synthase *in vitro* (Moore et al.,

1990; Rees et al., 1990) while L-NAME has the advantage of being more readily soluble. In contrast to the findings of Bhardwaj & Moore (1988) with less selective, presumed EDRF(NO) inhibitors, the present study with more selective tools therefore suggests that EDRF(NO) is not a mediator of vasodilation to histamine in the preconstricted isolated perfused rat kidney. The reason for the discrepancy between findings may concern a non-selective action of putative EDRF(NO) inhibitors or conceivably, methodological differences or a divergence in the mechanism(s) of renal vasodilation to histamine between the rat strains used. Evidence of significant NO synthase inhibition in the present study was provided both by the depression of vasodilation to acetylcholine (0.3nmoles) by L-NAME (0.3mM) in the methoxamine-preconstricted isolated perfused rat kidney and by the rise in perfusion pressure accompanying L-NOARG in potassium-preconstricted preparations.

Paradoxically and despite several reports that basal NO release regulates renovascular resistance in rats (Welch et al., 1991; Radermacher et al., 1990; Tolins et al., 1990), no elevation in perfusion pressure was observed with L-NAME (0.3mM). This fact may be attributable to the anomalously high preconstricted perfusion pressures observed in preparations to be exposed to L-NAME which, while allowing for the observation of vasodilator responses, might have functionally antagonised further

rises in tone due to the loss of an endogenous vasodilator. Conceivable alternative explanations are that reputed isoenzymes of NO synthase responsible for basal and agonist-stimulated EDRF(NO) release may be differentially affected by L-arginine analogues (Frew et al., 1993; Crack & Cocks, 1992) or that L-NOARG may raise tone by an action independent of the inhibition of NO synthase (Cocks & Angus, 1991). The present results with L-NAME are supported by Farhat et al. (1990) who reported the ability of another NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA), to inhibit endothelium-dependent vasodilation in the absence of an effect to raise tone in the α_1-agonist-preconstricted isolated perfused rat kidney.

The failure of indomethacin (10μM) to reduce vasodilation to histamine in the potassium-preconstricted isolated perfused rat kidney, additionally suggests that cyclo-oxygenase products may be ruled out as vasodilator mediators. Bhardwaj & Moore (1988) reached a similar conclusion with indomethacin in the α_1-adrenoceptor-preconstricted isolated perfused rat kidney. However, the vasodilator effect of indomethacin *per se* in the present study does argue for either a basal or depolarisation-stimulated release of a vasoconstrictor prostanoid, possibly prostaglandin E_2 (see Baer & McGiff, 1979).

The notion that vasodilation in the rat isolated perfused kidney may involve vascular H_3 receptors functionally coupled to EDRF(NO) and prostacyclin release, a mechanism previously described in the rabbit cerebral intracranial artery by Kim et al. (1992), is clearly refuted by the ineffectiveness of the H_3 receptor antagonist thioperamide in conjunction with the inability of the NO synthase inhibitors L-NAME and L-NOARG and the inability of the cyclo-oxygenase inhibitor indomethacin, to modulate renal vasodilation to histamine.

The endothelium as a potential source of vasodilator mediators other than EDRF(NO) and cyclo-oxygenase products, cannot be excluded by the present study. As an alternative explanation to endothelial involvement, histamine could elicit vasodilation via a direct action on histaminergic receptors on vascular smooth muscle to raise intracellular levels of cyclic adenosine monophosphate (cAMP) (Hill, 1987; Reinhardt & Ritter, 1979).

Taken together, the available evidence indicates that neither EDRF(NO) nor cyclo-oxygenase products act as mediators of the vasodilation to histamine in the preconstricted isolated perfused rat kidney and furthermore suggest that this vasodilator response is predominantly subserved by H_2 receptors.

4.2. Vasoconstriction to endothelins in the isolated perfused rat kidney: effects of BQ123 and indomethacin

Two main findings from the relatively simple isolated perfused kidney preparation *in vitro* support the contention by Cristol et al. (1993b) *in vivo* that renal vasoconstriction to ETs/SXs is mediated by a heterogeneous population of non-ET_A, possibly ET_B receptors in addition to ET_A receptors in the rat. Firstly, ETs were equipotent as vasoconstrictors in the isolated perfused rat kidney which is consistent with a family-common ET_B receptor-mediated event (Sakurai et al., 1990). The present report of equipotency of ETs as renal vasoconstrictors is in agreement with the findings of Evangelista et al. (1992). Secondly, a selective antagonist at ET_A receptors, BQ123 (Ihara et al., 1992a; Nakamichi et al., 1992), significantly depressed peak vasoconstriction to ET-1 at the lower doses. In addition, BQ123 significantly abbreviated the time to 75% recovery from peak vasoconstriction to ET-1 at the highest dose of ET-1 examined.

It is doubtful if BQ123 at a higher concentration than 1μM would have exerted any additional selective depression of the vasoconstrictor responses to ET-1. BQ123 is a potent ET_A receptor antagonist which displaces ^{125}[ET-1] binding to ET_A receptors in cultured porcine aortic smooth muscle membranes with an IC_{50} of 22nM

(Ihara et al., 1992a). ^{125}[ET-1] displacement is maximal at 1μM BQ123. Furthermore, the results of these displacement studies are in good agreement with the functional inhibition by BQ123 of the contraction of the porcine coronary artery to ET-1 (pA_2 7.4). As BQ123 begins to displace ^{125}[ET-1] binding to ET_B sites in porcine cerebellar membranes at concentrations greater than 1μM (Ihara et al., 1992a), the use of higher concentrations could result in non-specific actions and was therefore not justified.

The sensitivity of vasoconstriction to only the lower doses of ET-1 to BQ123 in the isolated perfused rat kidney, agrees well with the report by Cristol et al. (1993b) that renal vasoconstriction to higher doses of ET-1 in rats *in vivo* are insensitive to BQ123 while the responses to lower doses of ET-1 are diminished. The explanation provided by Cristol et al. (1993b) that the stimulation of ET_A receptors may be important in the mediation of a constrictor event to low doses of ET-1 but that an alternative, non-ET_A-mediated constrictor event may supervene at high doses, may explain the present findings.

BQ123 dramatically reduced the time to recovery from peak vasoconstriction to 30pmoles ET-1. Indeed, this parameter for 30pmoles ET-1 was reduced by some 70% after ET_A receptor blockade while the peak rise in perfusion

pressure was not significantly reduced (see Figures 13 & 14). This finding is evocative of the report by Bigaud and Pelton (1992) that the duration, but not the magnitide, of the renal vasoconstrictor response to the reputed ET_B-selective agonist [$Ala^{1,3,11,15}$]ET-1 (Saeki et al., 1991) is reduced by BQ123 in the anaesthetised rat. This clearly raises the possibility that an essentially BQ123-insensitive vasoconstrictor event can nonetheless be modified by an action at ET_A receptors. In the present study, the suggestion is that the occupation of ET_A receptors prolongs the duration of vasoconstriction to at least higher doses of ET-1. Indeed, the time to 75% recovery from comparable peak vasoconstrictor effects of ET-1 and ET-3 (both 30pmoles) was similar in the presence of BQ123. The shorter-lived vasoconstrictor response to ET-3 relative to ET-1 and ET-2 at least at higher doses and the insensitivity of its time to 75% recovery to BQ123 may well reflect the much lower affinity of ET-3 for ET_A receptors (Ihara et al., 1992a). These findings help to rule out suggestions that a differential release of a vasodilator such as PGI_2 (Trybulec et al., 1991) or any other vasodilator may play a role in the relatively short-lived duration of vasoconstriction to ET-3 (30pmoles). In further support of this, indomethacin failed to significantly modulate vasoconstriction to ET-1 or ET-3. It is however still possible that ET-stimulated prostanoid release could

occur in the absence of an influence on vasoconstrictor reactivity (Stier et al., 1992).

The transience of the vasoconstriction to bolus doses of ETs in the rat isolated perfused kidney is in contrast to the prolonged responses observed in the isolated perfused rat heart (Baydoun et al., 1989; Thompson et al., 1992). One possibility is that the renal metabolic activity of neutral endopeptidase 24.11 (enkephalinase) (Vijayaraghavan et al., 1990; Yamaguchi et al., 1992) and a distinct endothelin degradation enzyme (Deng et al., 1992) may provide an efficient mechanism for the biological modification of ETs and the reversal of their vasoconstrictor effects (see Kimura et al., 1988).

In summary, ETs acted as equipotent vasoconstrictors in the isolated perfused rat kidney and the peak vasoconstrictor effects to lower doses of ET-1 were significantly sensitive to BQ123 (1μM). 30pmoles ET-1 and ET-2 displayed a prolonged recovery from peak vasoconstriction relative to 30pmoles ET-3 and this disparity could be shown for ET-1 to be dependent on an action at ET_A receptors. Overall, the findings generally support the notion that renal vasoconstriction to ETs is subserved by a mixed population of ET_A and ET_B-like receptors.

4.3. Vasoconstrictor reactivity in the isolated perfused rat kidney after renal ischaemia and reperfusion in vivo

This study provides no evidence that renal vascular resistance is enhanced *in vitro* after 30min ischaemia or 30min ischaemia and 15min reperfusion *in vivo*. This is in contrast to reports by Arendshorst et al. (1975), Clozel et al. (1991) and Paller et al. (1984) *in vivo* and Lieberthal et al. (1989) *in vitro* suggesting a postischaemic renal vasoconstriction in the rat kidney. The present discrepancy with these findings may be explained by the more severe lengths of occlusive renal ischaemia employed in these studies (45 or 60min). However, the study of Lieberthal et al. (1989) in the isolated rat kidney perfused with erythrocyte-rich salt solution employed a similar ischaemic (nonperfusion) time of 25 min. The present findings are in agreement with those of Linas et al. (1988) who demonstrated no change in renal vascular resistance *in vitro* after 20 min occlusive renal ischaemia *in vivo* in the rat. The above observations are however consistent with postocclusive renal vasoconstriction being an occlusive time-dependent phenomenon.

Although the apparent depression of vasoconstriction to noradrenaline after 30min ischaemia *in vivo* did not attain statistical significance, the potency of noradrenaline displayed a significant recovery with time

in vitro. In contrast, no such shift in potency to noradrenaline was observed in kidneys subjected to 30min ischaemia and 15min reperfusion *in vivo*. The transient aberration of vasoconstrictor reactivity to noradrenaline is evocative of a stunning effect of reversible ischaemia on the renal microvasculature (see Bolli et al., 1988). These findings would seem to be specific for noradrenaline insomuch as no shift in potency to 5-hydroxytryptamine was observed over a similar perfusion period *in vitro* after 30min renal ischaemia *in vivo*. In addition, non-specific vasoconstrictor reactivity to KCl was not significantly altered in the ischaemic group. A depression in vasoconstriction to noradrenalnie has been noted by Kelleher et al. (1984) in the rat kidney during the recovery phase after noradrenaline-induced acute renal failure *in vivo* while Conger et al. (1991) have shown that vasoconstriction to noradrenaline is lost altogether after 75min ischaemia in the rat *in vivo*. This suggests that incremental periods of renal ischaemia *in vivo* may give rise to a graded loss of renal vasoconstrictor reactivity to noradrenaline. Indeed, Clozel et al. (1991) have reported a predominant renal vasodilator effect of noradrenaline *in vivo* after 45min renal ischaemia in the rat. There is some evidence to suggest that the apparent loss in vasoconstrictor reactivity to noradrenaline after ischaemia may involve a defect of the α_2-adrenoceptor (McGillivray-Anderson et al., 1991).

Renal vasoconstriction to ET-1 and ET-3 *in vitro* was unaffected by 30min renal ischaemia *in vivo*. This is in good agreement with a study by Clozel et al. (1991) showing that renal vasoconstrictor responses to ET-1 in the rat *in vivo* were relatively well preserved after 45min occlusive renal ischaemia. However, Clozel et al. (1991) also showed that vasoconstrictor reactivity to noradrenaline was abolished at this time. This was argued to favour a potential role for ET-1 in the observed postischaemic vasoconstriction in this study. An effect at the level of glomerular ET receptors accounting for the intact vascular reactivity to ET-1 after ischameia, would seem unlikely given the report by Wilkes et al. (1991) that neither the density nor affinity of these receptor sites change after up to 60min occlusive renal ischaemia in the rat. The present study would suggest that such marked differential changes in vasoconstrictor reactivity to noradrenaline and ET-1, together with a change in postocclusive renal vascular resistance either do not occur or are not sustained following a renal ischaemic time of 30 min *in vivo*. However, this period of reversible ischaemia may nevertheless be associated with temporary defects in renal vasoconstrictor reactivity, at least to noradrenaline.

4.4. Assessment of MPO activity in rat renal tissue after ischaemia/reperfusion in vivo: effects of antibodies against adhesion molecules used by PMNs

The present findings agree with those of Suzuki et al. (1983) that a TMB assay can provide a viable, safe alternative to the established o-dianisidine assay of MPO. A corollary of the enhanced sensitivity of the described TMB assay of MPO is that the threshold of detection is lowered. This theoretically provides for the assessment of early tissue PMN infiltration during reperfusion after ischaemia which might be overlooked using a less sensitive o-dianisidine assay (see Augustin & Lutz, 1991; Bird et al., 1988).

The masking of exogenous horseradish peroxidase activity by rat renal supernate indicates the presence of rat renal tissue factors that interfere with the peroxidase assay. Hillegas et al. (1990) have suggested that such factors may include conjugates of kidney-derived protein and the detergent HTAB, used to solubilise MPO. The level of inhibition (91%) exhibited by rat renal tissue assessed by the recovery of added horseradish peroxidase activity, was comparable to that reported by Schierwagen et al. (1990) (88%) measuring the recovery of exogenous human MPO activity in the rabbit kidney. However, in contrast to the findings of Schierwagen et al. (1990) in the rabbit kidney, the rat renal inhibition of exogenous

peroxidase activity was not abolished by heat inactivation alone. In fact, the level of rat renal inhibition remained significantly high at 49% after the rat renal supernate had been incubated at 60°C for 2h. The discrepancy between studies suggests the existence of both heat labile and heat stable interfering factors in rat renal tissue. The observation that rat renal inhibition was effectively abolished by the provision of excess peroxide to heat-inactivated rat renal supernate implies that the proposed heat stable factors are peroxide consuming systems. A conceivable role for such systems was postulated by Schierwagen et al. (1990). It is unlikely that the systems are themselves peroxidatic since no activity was detected in the renal supernate of non-ischaemic rat kidneys *per se*. Hence, tissue catalatic activity may be responsible. However, one prime candidate, the heat stable enzyme catalase, can probably be excluded since DMSO at 10% (v/v), which was present in all TMB assays, has been reported to abolish catalase activity *in vitro* (Rammler, 1967). Schierwagen et al. (1990) reports no significant loss of human MPO activity after incubation at 60°C for 2h. The present determination of MPO from rat PMNs undergoing the same treatment similarly attests the heat stability of rat MPO.

The applicability of procedures designed to optimise the recovery of exogenous peroxidase activity from renal

supernate *in vitro*, was demonstrated by the delineation of a time course for renal PMN accumulation using MPO as a biochemical marker after renal ischaemia/reperfusion *in vivo*. As further verification, no MPO activity could be detected in ischaemic/reperfused kidneys when the level of renal inhibition of added peroxidase activity was 91%. This emphasises the importance of assessing and overcoming the renal suppression of peroxidase activity before attempting ischaemia/reperfusion studies of renal PMN accumulation using MPO as a biochemical marker. Interestingly, Willinger et al. (1992) have histologically demonstrated PMN accumulation predominantly at intraglomerular, outer medullary and cortical peritubular sites in the rat kidney after 45min ischaemia and 2h reperfusion *in vivo*.

The conservative dose of MoAbs used in the present study (40μg/kg i.v. total) was selected entirely on economic grounds. A preferred approach is to first examine the blocking efficacy of anti-adhesion molecules in *in vitro* models of PMN endothelial adherence such as those described by Argenbright et al. (1991) and Polhman et al. (1986), and then to select a dose accordingly for *in vivo* use that would provide a similar concentration and presumably a similar blocking activity *in vivo*.

In the present study, the efficacy of MoAbs against ICAM-1 and CD18 of $ß_2$-integrins to block renal PMN

accumulation after ischaemia/reperfusion, would suggest the involvement of these adhesion molecules *in vivo*. This would agree with previous studies *in vitro* indicating the importance of ICAM-1 and CD18 to PMN-endothelial cell adhesion (Argenbright et al., 1991; Pohlman et al., 1986). However, the present results must be qualified by the findings that the isotype-matched control murine antibody, IgG_1 in addition to an unrelated murine antibody, IgG_{2a}, reproduced this blockade of renal PMN accumulation.

One possibility worth investigating is that murine antibodies may reduce the circulating level of PMNs. It is conceivable that endogenous rat anti-murine isotype antibodies could form immune complexes with exogenous murine antibodies and thereby precipitate PMN priming or activation, possibly via complement activation. This could lead to neutropaenia due to reduced PMN deformability and the subsequent sequestration of PMNs in the pulmonary vascular bed (MacGregor, 1977; Worthen et al., 1989; Jagels & Hugli, 1992).

There was no evidence that exogenous horseradish peroxidase activity was inhibited by renal supernate exposed to the isotype-matched control murine antibody, IgG_1. This indicates that murine antibodies do not directly affect tissue MPO activity *in vitro*. Taken overall, this suggests that murine MoAbs against rat

adhesion molecules may be capable of depressing ischaemic/reperfused renal PMN accumulation at least in part by virtue of a non-specific action *in vivo*. FACS analysis which showed the selective binding of anti-CD18 MoAb to rat blood PMNs, implies that a common, non-specific action of murine antibodies at the level of the PMN membrane can be excluded. However, non-specific binding by other cell types such as endothelial cells cannot be discounted. The level of PMN accumulation in ischaemic/reperfused kidneys exposed to the vehicle (0.9% saline) alone was not significantly reduced, indicating that effects were related to antibody interventions.

The FACS data is consistent with the surface expression of CD18, but not ICAM-1, by PMNs. This could be further verified by performing a FACS anaysis on activated PMNs which are known to display an upregulated surface expression of CD18 (Albelda & Buck, 1990). In this case, the density of anti-CD18 MoAb binding would be expected to be enhanced.

The gain in wet weight of ischaemic/reperfused kidneys indicates oedema formation. This occurred in the presence and absence of demonstrable ischaemic/reperfused renal PMN accumulation. This suggests a largely PMN-independent rise in renal microvascular permeability in this model of renal ischaemia/reperfusion (Wedmore and Williams, 1981; Williams et al., 1990). Oedema-promoting factors linked

with coagulation (Gerdin and Saldeen, 1978) may be excluded since animals were heparinised. Roles for local autocoids and/or vascular permeability factor (Collins et al., 1993) are conceivable. Alternatively, the integrity of the endothelial barrier may have been irreversibly damaged by ischaemia/reperfusion.

In summary, it has been found that a TMB assay provides a more sensitive photometric assessment of rat PMN-derived MPO than an established o-dianisidine assay. It was also determined that rat renal tissue factors which interfere with the assessment of rat renal tissue peroxidase activity could be surmounted by a combination of heat inactivation and the provision of excess assay H_2O_2. Renal PMN accumulation after ischaemia/reperfusion *in vivo* using MPO as a biochemical marker in a sensitive TMB assay, was subsequently demonstrated. Finally, the available evidence suggests that murine MoAbs against rat adhesion molecules may be effective in reducing renal PMN accumulation after ischaemia/reperfusion *in vivo* at least in part in a non-specific manner.

4.5. Conclusions

Histamine is not an EDRF(NO)-dependent vasodilator in the isolated perfused rat kidney. Histamine would therefore not be a suitable agent in an assessment of agonist-

elicited renal EDRF(NO) function after ischaemia/reperfusion in the rat. The predominant receptors mediating renal vasodilation to histamine in the rat were determined to be H_2 receptors. Vasoconstriction to endothelins in the isolated perfused rat kidney is mediated by a mixed population of ET_A and non-ET_A, possibly ET_B receptors. The ET_A receptor plays a role in prolonging the duration of vasoconstriction to ET-1 (and probably ET-2). Basal renal vascular resistance and vasoconstrictor reactivity to ET-1, ET-3, noradrenaline, 5-hydroxytryptamine and KCl is not grossly affected *in vitro* following 30min ischaemia with or without 15min reperfusion *in vivo*. However, this period of ischaemia *in vivo* is sufficient to reversibly depress the renal vasoconstrictor potency of noradrenaline *in vitro*. Finally, rat renal tissue is capable of suppressing peroxidase activity. This suppression is surmountable by a combination of heat inactivation of the renal tissue and the provision of a suitably high level of peroxide in the assay mixture. Myeloperoxidase, a biochemical marker for polymorphonucleocyte accumulation, is detectable in rat kidneys after ischaemia/reperfusion *in vivo*. The earliest observed renal PMN accumulation occurred after 45min ischaemia and 1h reperfusion. Murine monoclonal antibodies against rat adhesion molecules ICAM-1 and CD18, may be able to reduce renal PMN accumulation after ischaemia/reperfusion in a non-specific manner in the rat.

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